

REMARKS

Applicants respectfully request reconsideration of this application in view of the following remarks.

I. Status of the Claims

Claims 1 and 44-99 remain pending in the application, and are not amended. Claims 1, 44-55, 63-76 and 84-85 are under examination, while claims 56-62, 77-83 and 86-99 are withdrawn from consideration.

II. § 112, First Paragraph, Rejections

The claims were rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of support for the exclusion of alum. The Examiner alleges that the portions of the specification disclosing alum as an optional component do not support the exclusion of alum, but this position is directly contrary to the MPEP. As pointed out in Applicants' previous response, MPEP § 2173.05(i) explains that, "[i]f alternative elements are *positively recited* in the specification, they may be *explicitly excluded* in the claims" (emphasis added). Thus, the language excluding alum in claims 1 and 64 is fully supported by the application as filed, for example, at page 26, line 19. Applicants therefore respectfully request reconsideration and withdrawal of the §112 rejections.

III. The Claimed Invention is Novel

Claims 1, 44, 46-55, 64, 66-76 and 85 were rejected as allegedly anticipated by Simmonds A (WO 94/25602; §102(b)); Simmonds B (U.S. Patent 6,881,821; § 102(e)); or Simmonds C (U.S. Patent 7,198,892; §102(e)), in light of teachings by Sjolander (1998). Applicants respectfully traverse these rejections.

The rejected claims are directed to "an immunogenic complex comprising a negatively charged organic complex and a charged antigen, which organic complex and antigen are electrostatically associated," and wherein the charged antigen comprises one or more polypeptides from a specified region of HCV, and to compositions comprising such a complex. This subject matter is not taught or suggested by the cited references.

Simmonds A-C are directed to “a previously unknown type 6 variant of HCV,” and to peptides that are described as being type-specific to HCV-4, HCV-5, and HCV-6. Thus, the focus of Simmonds A-C is the description of these peptides *per se*, not their formulation into novel immunogenic complexes, as claimed. The only portion of Simmonds with any relevance to the present invention appears to be that cited in the Office Action, i.e., page 24 of Simmonds A, column 10 of Simmonds B, and column 11 of Simmonds C. As noted by the Examiner, these portions of Simmonds state that the disclosed peptides “may optionally be **attached** to a particulate structure, such as liposomes or ISCOMS” (emphasis added). This teaching, however, does not anticipate the present invention, because it only suggests “attaching” the peptides to an ISCOM, not preparing an **electrostatically associated** complex, as recited in the instant claims.

As explained previously, in the claimed complex, there is an electrostatic association between the negatively charged organic complex and charged antigen, which involves an ionic bond. There is no teaching or suggestion in Simmonds A-C of forming an electrostatic association between its peptides and ISCOMs via ionic bonds, or other electrostatic means. To the contrary, as of the 1994 filing date of Simmonds A-C, those skilled in the art would have understood the teaching of peptides “attached to a particulate structure, such as liposomes or ISCOMS,” to implicate non-ionic bonding. For example, Barr et al., *Adv. Drug. Deliv. Rev.* 32: 247-71 (1998) (copy attached), discusses the incorporation of non-amphipathic molecules (*e.g.*, peptides and proteins) into ISCOMS in section 4.2, at page 254. The authors note the difficulty of doing so, but cite Lovgren et al., “Antigenic Presentation of Small Molecules and Peptides Conjugated to a Preformed Iscom as Carrier,” *J. Immun. Meth.* 98: 137-43 (1987) (abstract attached), for reporting methods of “chemically coupling peptides . . . onto preformed . . . ISCOMs.” Thus, the skilled artisan reviewing Simmonds likely would have understood the reference to peptides “attached to . . . ISCOMS,” to refer to covalent attachment, as illustrated in Lovgren and Barr.

For at least these reasons, Simmonds A-C fail to teach a complex or composition meeting every limitation of the rejected claims. Thus, the §102 rejections are improper and should be withdrawn.

IV. The Claimed Invention is Non-Obvious

Claims 1, 44-55, 63-76 and 84-85 were rejected under 35 U.S.C. § 103(a) as allegedly obvious over Simmons A-C in combination with Cerny (1995). Applicant respectfully traverses these rejections.

The inability of Simmons A-C to teach or suggest the claimed invention is shown above. While Cerny is cited for teaching HCV T-cell epitopes, combining Simmons A-C with Cerny does not resolve Simmond's inability to teach or suggest the claimed immunogenic complexes. In particular, combining Simmons with Cerny still leaves the skilled artisan with no guidance to form "an immunogenic complex comprising a negatively charged organic complex and a charged antigen," wherein the "organic complex and antigen are electrostatically associated." Because no cited combination of references teaches or suggests such an immunogenic complex, the §103 rejections are improper and should be withdrawn.

IV. Concluding Remarks

Applicants believe that this application is now in condition for allowance, and an early notice to that effect is earnestly solicited.

Should there be any questions regarding this submission, or should any issue remain, the Examiner is invited to contact the undersigned attorney by telephone in order to advance prosecution.

The Commissioner is hereby authorized to charge any additional fees that may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extensions under 37 C.F.R. §1.136 and authorize payment of any extension fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date October 3, 2007

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 672-5475
Facsimile: (202) 672-5399

By Beth A. Burrous
Reg No 37,288
Beth A. Burrous
Attorney for Applicant
Registration No. 35,087

Attachments



ISCOMs and other saponin based adjuvants

I.G. Barr^{*}, A. Sjölander, J.C. Cox

CSL Limited, 45 Poplar Road Parkville, Melbourne, Victoria 3052, Australia

Received 18 August 1997; received in revised form 22 September 1997; accepted 18 October 1997

Abstract

Saponins are chemically a heterogeneous group of sterol glycosides and triterpene glycosides which are common constituents of plants. One source of triterpenoid saponins obtained from the bark of *Quillaja saponaria* Molina (the soap bark tree) have been known to cause substantial enhancement of immune responses since the 1920s. Despite their use in animal vaccines, the development of saponin-based formulations for human vaccines has been impeded by their complexity and concerns about toxicity. This review briefly covers the use of saponins for animal vaccines but focuses mainly on the development of these adjuvants for use in man. Important aspects include preparation of purified or highly defined saponin fractions, improved understanding of the relationships between adjuvant activity, toxicity and structure of saponins and formulation of saponins into structures with reduced toxicity such as ISCOMs. Recent developments in the understanding of cellular interactions, cytokine induction and the in vivo localisation of saponin containing formulations will also be reviewed. © 1998 Elsevier Science B.V.

Keywords: *Quillaja saponaria*; Triterpene glycosides; QS-21; ISCOPREPTM703; Cytokines; Vaccine

Contents

1. Introduction	248
2. Source, purification and formulation of saponins	248
3. Structural and functional analysis of purified <i>Quillaja</i> saponins	250
3.1. Branched carbohydrate at the C3 position	251
3.2. The carbohydrate chain at C28	251
3.3. The nature of attachment of the acyl chain to fucose	251
3.4. The length of the acyl chain	251
3.5. Carbohydrate moieties on the acyl chain	251
3.6. The active aldehyde at C4 on the quillaic acid	252
4. ISCOMs	252
4.1. Incorporation of amphipathic molecules into ISCOMs	252
4.2. Incorporation of non-amphipathic molecules into ISCOMs	253
5. Other saponin based vaccine formulations	254
5.1. Liposomes	254
5.2. Microspheres	254
5.3. Aluminium salts	255
6. Mode of action	255
6.1. Uptake and distribution	255
	256

^{*}Corresponding author. Tel.: +61 3 93891761; fax: +61 3 93811247; e-mail: ibarr@csll.com.au

6.2. Antigen presentation	256
6.3. Cytokine induction	257
6.4. CTL induction	259
7. Toxicity	260
8. Studies of saponin based vaccine formulations in animal models	261
9. Vaccination in the presence of maternal antibodies	262
10. Veterinary vaccines using saponin based formulations	263
10.1. ISCOVAC FLU vet TM	263
10.2. Quilvax TM programs	263
11. Clinical testing	263
12. Conclusions	264
Acknowledgements	264
References	264

1. Introduction

Saponins are a heterogeneous group of sterol glycosides and triterpenoid glycosides that are present in a wide range of plant species where they are distributed throughout the bark, leaves, stems, roots and even flowers [1]. While their basic function in plants is unknown, they are known to inhibit mould and protect plants from insect attack. In addition, saponins are anti-microbial and can be a source of monosaccharides [2]. Saponins have long been known to possess properties useful to man and were used by Australian Aborigines to harvest fish and snails [3]. Triterpenoid saponins are still widely used commercially for their detergent properties as cheap foaming agents ('sapon' = soap) and, in a return to one of their more traditional applications, saponins are used in Africa to kill infected snails and prevent the transmission of schistosomiasis [4]. It was Espinet's [5] inclusion of saponin into a veterinary vaccine in the 1950s that revived the use of saponin as an adjuvant. A closer analysis of Espinet's work by Dalsgaard [6] revealed that the only source of saponin which was effective in the trials had been obtained from the bark of the *Quillaja* (also spelt *Quillaja*) *saponaria* Molina tree (triterpene saponins). Since this time a number of purification methods have been applied to crude *Quillaja* saponins in an attempt to separate the adjuvant active components from the toxic ones. Another approach to overcoming the toxicity has been the incorporation of the saponin into lipid particles known as Immuno-stimulating Complexes or ISCOMs [7].

Trends in modern vaccine development towards the use of highly purified recombinant proteins,

which are often poorly immunogenic when given alone or in combination with aluminium salts, have driven research on saponin adjuvants. Also, there is a need with some vaccines to induce an immune response capable of eliminating virus-infected or malignant cells. This is an activity which saponin based adjuvants, but few others, appear to possess. This feature is due to the ability of saponins to stimulate the cell mediated arm of the immune system as well as enhancing antibody production. The focus of this review will be the recent work on ISCOMs, QS-21 and other saponin based adjuvants with an emphasis on the recent developments including their mode of action. For other reviews of ISCOMs and QS-21, the reader is directed to the following references [8–13].

2. Source, purification and formulation of saponins

Saponin is commercially obtained from four major sources: *Smilax ornata* (sarsaparilla), *Gypsophilla paniculata* (brides veil), *Saponaria officinalis* (soap root) and *Quillaja saponaria* Molina (soap bark). The saponin content of bark is approximately 5% by weight. Extraction of the bark with aqueous solutions yields a colored mixture of saponins, polyphenols and tannins. In the case of *Quillaja saponaria* Molina, saponins represent only 20–25% of the extractable material [11]. Confusion with respect to the source of the saponin and the variable content of saponin in extracts, explains much of the reported variation in adjuvant activity. In an effort to overcome this variability, Dalsgaard [14] utilized anion

exchange and gel filtration to purify *Quillaia* saponins into a fraction he termed Quil A, which is highly adjuvant active. This process was subsequently developed commercially by Superfos (Denmark) and this purified material is used in a number of veterinary vaccines [15]. Quil A was standardized for triterpenoid saponin content and had predictable adjuvant activity with less local toxicity in cattle than an equivalent amount of crude saponin [14].

While being suitable for veterinary applications, Quil A was less satisfactory for human applications as it, and other similar preparations, were found to be a heterogeneous mixture of saponins when analysed using High Performance Thin Layer Chromatography (HP-TLC) and Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Three classes of saponin were identified by Kartnig and Ri [16], seven by Higuchi et al. [17], 17 by Marharaj et al. [18], 22 by Kensil et al. [19,20], 23 by Kersten et al. [21,22] and 30 by So et al. [23]. The existence of many different saponins, which vary in their chemical or biological activities, makes characterisation of the material difficult and more importantly, may lead to unpredictable effects in vivo as a result of the variable content of the individual saponins in the mixture.

Kensil [19,20] identified adjuvant activity in 10 of the fractions tested, including the four most abundant saponins, termed QS-7, 17, 18 and 21; the numbers correspond to their relative elution time which is dependant on their degree of hydrophobicity using C_{18} resin with RP-HPLC. When these fractions were tested with bovine serum albumin (BSA) and cytochrome b_5 for their ability to enhance serum antibody levels, equally high titres were obtained for QS-7, 17, 18 and 21. There were, however, some differences between QS-7 and QS-17, 18 and 21 in the levels of mouse IgG subclasses induced.

Chemical and physical analysis showed that these major fractions of crude saponin had molecular weights of between 1800 and 2200 with their size varying upon the particular composition of monosaccharides. By fast atom bombardment mass spectroscopy (FAB-MS), the ion masses of QS-17, 18 and 21 were 2321, 2174 and 2012 respectively [24]. Similarly, Higuchi et al. [25] calculated the molecular weight of QSHI, which is equivalent to QS-17, as 2296 from which the molecular weights of QS-18

and QS-21 can be calculated as 2150 and 1988 respectively. Kersten [22] claimed that all 23 fractions separated by RP-HPLC using C_{18} resin had adjuvant activity. Morein et al. [26] described the purification of three saponin components to high purity. These fractions, which he designated B2, B3 and B4B with molecular masses of 1988, 2150 and 1862 respectively, are probably equivalent to QS-21, QS-18 and QS-7. While all three molecules bound cholesterol, only B4B formed typical ISCOM-like particles. The B3 fraction was found to be the most potent as an adjuvant and B4B the least potent [26].

In studies carried out by Rönnerberg et al. [27], the characteristics of three different fractions of Quil A were examined, after purification by RP-HPLC. One sequence of fractions was eluted early and was termed QH-A while a further two sequences of the more hydrophobic fractions were eluted later and designated as QH-B and QH-C. Adjuvant activity was determined by subcutaneous injection of mice with 10 μ g of QH-A, B or C mixed with 1 μ g of A/PR8/34 influenza micelles (flu-m). All fractions were adjuvant active with the highest titres of antibody induced using the QH-B or QH-C while QH-A was somewhat less potent. This study was followed by pre-clinical toxicology testing and animal testing using a combination of seven parts QH-A, 0 parts QH-B and three parts QH-C, material known as QH-703 or ISCOPREP™703, (Isotec AB, Sweden). This mixture may have been developed to mimic the natural ratios of QH-A:QH-C found in Quil A while excluding the more toxic QH-B components. This mixture was found to be adjuvant active and showed enhanced Interleukin 1 α (IL-1 α) production by murine peritoneal cells, whereas other QH combinations were less active [28]. A list of the most studied purified saponins, their synonyms and some of their features are summarised in Table 1.

Two interesting alternatives to the use of bark from mature *Quillaia saponaria* Molina trees have been suggested by Dalsgaard [29]. The first suggestion was the use of young plants, less than 15 years old, for saponin purification as these extracts were found to be less heterogeneous than those obtained from mature bark extracts. A second suggestion was the use of in vitro plant cell cultures of *Quillaia saponaria* Molina cells as a source of

Table 1
Summary of the purified saponins terminology and features

Name	Synonym	Molecular weight	Features	References
QSIII	QS-17	2296	Adjuvant active	[19,25]
QS-7	B4B	1862	Adjuvant active, forms ISCOMs, very low toxicity	[19,26]
QS-18	Quadri 1, B3	2150	Adjuvant active, forms ISCOMs, toxic.	[19,26,29]
QS-21	Quadri 2, B2	1988	Adjuvant active, forms ISCOMs, low toxicity	[19,26,29]
DS-1	QS21H, Quadri2A	1590	Obtained by mild alkaline hydrolysis, low adjuvant activity	[17,20,29]
DS-2	QS18H, Quadri 1A	1752	Obtained by mild alkaline hydrolysis, low adjuvant activity	[17,20,29]
QS-957	Quadri 1B or 2BQS-L1	957	Obtained by strong alkaline hydrolysis, low/no adjuvant activity	[20,23,29,36]

saponins. This opens the possibility that saponins for human therapeutics may one day be derived from a defined master cell bank rather than being dependent upon natural sources, although the commercial viability of this process has yet to be established.

3. Structural and functional analysis of purified *Quillaia* saponins

The chemical structures of the *Quillaia* saponins are highly complex with many opportunities for

diversity. RP-HPLC analysis has revealed up to 30 components in preparations of *Quillaia* saponins such as Quil A [23]. It is likely that the true number of variants would exceed 100 if all conformational isomers were produced. The first complete structural analysis of a *Quillaia* saponin was carried out by Higuchi et al. 1988 [25] for a molecule they termed QSIII (Fig. 1). This molecule is now known to be identical to QS-17 [30] as described by Kensil et al. 1988 [20] based on chromatographic and carbohydrate analysis. *Quillaia* saponins have a five-ringed quillaic acid backbone with small carbohydrate

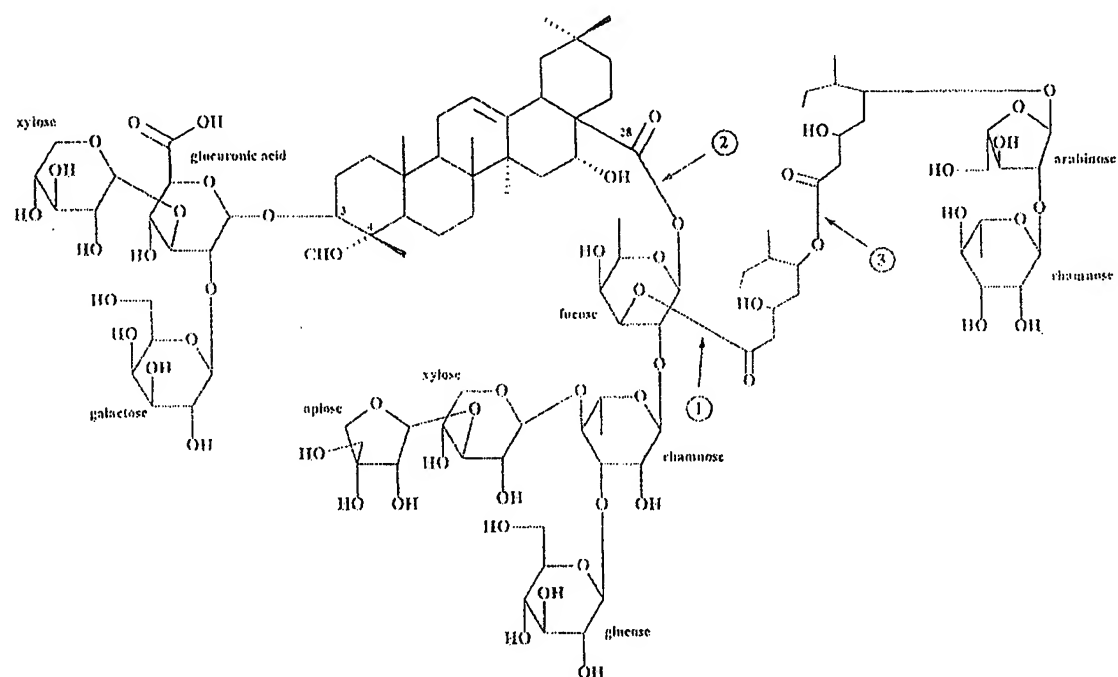


Fig. 1. Proposed structure of QSIII (QS-17) adapted from Higuchi et al. [25].

chains, consisting of two to five sugar units, attached at the 3' and 28' carbons of quillaic acid and are frequently branched [31]. Attached to the fucose first sugar unit at the 28' position of the carbohydrate chain is an 18 carbon acyl chain with a small carbohydrate chain, consisting of one or two sugar units, at its terminal end. The major areas of chemical diversity are listed in the following 6 subsections.

3.1. Branched carbohydrate at the C3 position

Until recently, this position was considered constant. However Guo et al. [32] identified two new structures where xylose is either absent or replaced with rhamnose. These were isolated from QH-A or from a mixture of QH-A and QH-C after strong alkaline hydrolysis. The three variants were present in approximately equal quantity. Although the disaccharide could possibly be argued to be a breakdown product, the replacement of a pentose with a deoxyhexose must occur during synthesis. Modification of the carboxyl group of the glucuronic acid [33] caused a minor to substantial effect upon adjuvant activity, depending upon the nature of the substitution. It appeared possible that the decrease in adjuvant activity was greater as the charge changed from negative to neutral to positive as a result of this chemical modification. The effect of these changes on hemolytic activity to sheep red blood cells (SRBC) was small.

3.2. The carbohydrate chain at C28

Considerable variation has been reported in the carbohydrate chain at C28 of the quillaic acid. Kensil et al. [34] and Sotysik et al. [35] identified QS-21-V1 and QS-21-V2 as two different compounds which co-purified by RP-HPLC [20] but could be separated by hydrophilic interaction chromatography. Each has a tetra-saccharide chain with terminal apiose and xylose respectively and exist in a ratio of 2:1 [36]. Both have similar adjuvant activity [34]. Similar diversity was reported by Dalsgaard [29] for Quadri 2, a saponin fraction thought to be identical to QS-21. In addition, he observed two other molecular species from this HPLC fraction, one with a trisaccharide at the C28, Quadri 2-tri, and another uncharacterized.

The other region of variation involves the addition of glucose in 1→3 configuration to rhamnose to create a branched chain which is present in QS-18, equivalent to Quadri -1, and QS-17, equivalent to QSIII. This single addition to the molecular structure leads to a substantial (at least six-fold) increase in lethal toxicity in mice [19,29] but a two-fold reduction in hemolytic activity [19]. Adjuvant activity however was little changed by this modification [19,29].

3.3. The nature of attachment of the acyl chain to fucose

This was initially identified [25] as a 3' attachment for QSIII and subsequently presumed to also apply to QS-18 and QS-21 [24]. More recently [30] two regioisomers, QS-21A and QS-21B with 4' and 3' attachment respectively have been described. Although these two isomers can be separated by RP-HPLC, they rapidly re-equilibrate to a ratio of A:B variously reported as 20:1 [30] or 4:1 [36], equilibration being complete after 100 min at 37°C, pH 7.4. Commercial QS-21 is QS-21A. Both isomers are reported to be of similar adjuvant activity but the rapid isomerisation prevents meaningful measurement of this property [36].

3.4. The length of the acyl chain

Higuchi et al. [17] prepared a semi-purified *Quilaidia* saponin mixture, which showed seven spots by HP-TLC, and subjected this to mild alkaline hydrolysis to yield two products – DS1 and DS2, which were subsequently shown to be identical to QS-21H, or Quadri-2A, and QS-18H, or Quadri-1A, respectively [20,29]. These structures result from ester hydrolysis at position 1 (Fig. 1), and differ only by the presence, or absence, of glucose. DS-1 and DS-2 can also be identified in Quil A [29]. In limited studies [20] deacylation was shown to substantially reduce the adjuvant activity of QS-18 and QS-21. Strong alkaline hydrolysis of either DS1 or DS2 [17] yielded a single compound by hydrolysis at position 2 (see Fig. 1), which is identical to Quadri-1B and 2B [29] and QS-957 [37] and QS-L1 [23]. This molecule is generally considered to lack adjuvant activity although So et al. [23] demonstrated activity in synergy with alum. They suggested the role of the

acyl chain may be to bind or associate with antigens through hydrophobic interactions.

Base-catalysed ester hydrolysis has been shown to occur at pH above 7.0 and the shelf life at pH 7.4 and 25°C was estimated to be 24 days [36]. QS-21 was more stable above its critical micellar concentration (CMC) presumably because the ester group was hidden. Similar studies with Quadri-2 ISCOMs have shown no alkaline hydrolysis occurred even at pH 9.0 (K. Dalsgaard, personal communication), presumably because the susceptible ester bond is hidden. As acid catalysed ester hydrolysis can occur below pH 4.0, Cleland et al. [36] recommended storage at pH 5.5.

The chemical characterisation of QS-7 has not yet been described. However its molecular weight of around 1870 [34], substantially reduced hydrophobicity [19] and lack of arabinose [20] suggest it may be a partially deacylated QSIII or QS-18 with ester hydrolysis at position 3 (Fig. 1). Hydrolysis of this ester bond was described by Higuchi et al. [17]. A fraction containing molecules of related hydrophobicity defined as QH-A [27] has been shown to form ISCOMs [38] a property expected to require a hydrophobic region. This expectation is supported by the observations of Dalsgaard et al. [29] where Quadri-1A formed smaller ring like complexes which did not seem to self assemble into the larger 40 nm ISCOM structures, which will be discussed in Section 4.

3.5. Carbohydrate moieties on the acyl chain

QS-17 has two carbohydrate molecules, QS-18 and QS-21 have one whilst QS-7 lacks any carbohydrate on the acyl chain. All four have similar levels of adjuvant activity [19] suggesting that these modifications have no role in promoting adjuvant activity.

3.6. The active aldehyde at C4 on the quillaic acid

Soltysik et al. [33] found that chemical modification of the aldehyde group on the quillaic acid caused an increase in the CMC and totally abolished hemolytic activity of SRBC. As hemolysis could occur below the CMC with unmodified saponins, they concluded that hemolysis was the function of

the monomer but required the ability to form micelles. Adjuvant activity was also abolished and it was suggested that the aldehyde may be involved in a Schiff's base interaction with free amino groups on a cellular membrane protein or lipids of lymphocytes or cells involved in immune responses [33]. Such interactions have been described between T cells and antigen presenting cells [39,40].

One further structure-function modification warrants consideration. Kensil et al. [24] showed that periodate modification eliminated adjuvant activity in QS-18, possibly forming a bridge between t-galactose and t-apiose. The possibility of stable intramolecular rearrangements of *Quillaia* saponins has been raised [29]; it was suggested such internal rearrangements occur spontaneously and might modify both toxic and adjuvant active properties of saponins.

A better understanding of the relationship between structure and function will increase the confidence involved with the formulation and storage of purified saponin containing vaccines. A list of the recommended storage conditions for Quil A, ISCOPREP™703 and QS-21 were given in Vogel and Powell [41]. For example the optimal storage of solid QS-21 was recommended to be -20°C in the dark with low humidity, while for aqueous solutions of QS-21 a range of pH 5.5–6.0 was desirable to minimise the ester hydrolysis of the acyl chain. Freezing of QS-21 containing solutions [42] or freeze drying of ISCOMs [43] have also been used successfully to minimise degradation of saponins with prolonged storage.

4. ISCOMs

Novel saponin containing particles, known as ISCOMs, have been developed (ISCOM and ISCOMS are trademarks of Iscotec AB) as an alternative approach for controlling potential toxicity. Saponins had long been known to interact with lipids in cell membranes forming complexes that lead to cell lysis [44–48]. In 1971 the first ISCOM-like structures were visualised by electron microscopy in virus preparations that had been treated with saponin in an attempt to isolate viral subunits, but these structures were regarded as artefacts [8,49]. Morein

et al. [7,49] first described a method for incorporating viral membrane components into saponin-containing complexes and purifying the particles to produce well defined, highly immunogenic particles for which the term ISCOM was given.

Two types of ISCOMs have been described in the literature. The first is the classic ISCOM, formed by the combination of cholesterol, saponin, phospholipid and viral envelope proteins [7] and the second is known as ISCOM matrix, also called empty ISCOMs or ISCOMATRIXTM [13]. Both forms are made identically with the exception that no protein is added to the ISCOM matrix but they still retain the basic ISCOM structure. ISCOMs and ISCOM matrix are typically rigid, hollow, spherical, cage-like particles which are negatively charged and measure about 40 nm in diameter [10,13,20,21,49,50]. The basis of the unique structure of ISCOMs is the interaction between saponin and cholesterol. Cholesterol is an essential component of ISCOMs and ISCOM matrix as is phospholipid with both phosphatidylethanolamine [21] or more commonly the egg derived phosphatidylcholine [51] having been used. The added phospholipid has been suggested to act by providing a looser 'fit' than cholesterol alone to allow amphipathic molecules, such as viral membrane proteins, to insert into the ISCOM [51]. The arrangement of the cholesterol-saponin micelles into the ISCOM particle have variously been described as a pentagonal dodecahedron arrangement of micelles, composed of lipid and saponin [50] or as a 'soccer ball' arrangement with the multiple-micelles held together by hydrophobic interaction, steric factors and possibly hydrogen bonds [52].

While many of the studies utilising ISCOMs have used Quil A as a source of saponin [7,8,13] a recent study examined the properties of purified saponins [27] both in their free form and after they were incorporated into ISCOMs. ISCOM matrix was made from QH-A, QH-B, QH-C or with various ratios of the purified QH fractions such as 3:6:1 (QH-A, QH-B, QH-C), 1:1 (QH-A:QH-C), 3:7 (QH-A:QH-C). The various types of ISCOM matrix were tested in mice for immunogenicity by adding 25 µg of ISCOM matrix to 1 µg of A/PR8/34 influenza protein. All combinations of ISCOM matrix enhanced the antibody response equally. ISCOMs and

ISCOM matrix using a QH-A:QH-C ratio of 7:3 (ISCOMPREPTM703) have also been included in human pre-clinical and Phase I trials, which will be described in Sections 7 and 11.

While both ISCOMs and ISCOM matrix are immunostimulatory, there may be an advantage for some vaccines to incorporate particular molecules, either naturally through an intact transmembrane domain or by chemically or physically altering the protein structure to obtain insertion/association [53–56]. Possible advantages of associating proteins with ISCOMs include partial purification or enrichment of membrane bound molecules, correct presentation of proteins for recognition, increased uptake of the protein-ISCOM structure [20,53–57] and CTL induction [38].

4.1. Incorporation of amphipathic molecules into ISCOMs

ISCOMs have been made with amphipathic molecules derived from membranes of a variety of viruses such as herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), Epstein-Barr virus (EBV), hepatitis B virus (HBV), rabies virus and influenza viruses. Cell wall proteins from bacteria, such as *E. coli* and *Brucella abortus* and parasites, such as, *Plasmodium falciparum* and *Toxoplasma gondii*, have also been formulated into ISCOMs [9,12,56,57]. When ISCOMs incorporate antigens derived from purified organisms or cell membranes, the molecules that are incorporated into ISCOMs are the proteins or glycoproteins which are normally anchored by a hydrophobic transmembrane sequence into the cell or viral membrane [20,43,55–58]. These molecules are extracted by detergent treatment and are integrated into the lipid-Quil A matrix and, when the detergent is removed, ISCOM particles are formed [51,57]. There have been situations where unexpected selective incorporation of a particular membrane protein into ISCOMs has occurred [55]. For example, the F glycoprotein of measles virus incorporated well into ISCOMs and liposomes in contrast to the H glycoprotein which was poorly incorporated into ISCOMs but well into liposomes. Interestingly, a good antibody response was generated to both the F and H glycoproteins following immunisation with the measles-ISCOMs [55].

ISCOMs have been formed with single and multiple amphipathic incorporated molecules [55,58–60] but, regardless of the range of different molecules, their size, level of glycosylation or tertiary structure, the ISCOMs that are generated are physically similar. In fact they are usually indistinguishable from ISCOM matrix when examined by electron microscopy [13,42,43,49,51,61] and have a similar sedimentation rate in sucrose gradients [51]. An exception is ISCOMs containing high levels of influenza hemagglutinin which can have spikes radiating from the surface of the ISCOM which are visible using electron microscopy and are presumably hemagglutinin molecules [9]. The degree of incorporation is usually determined indirectly by the co-incidence of the selected protein and ISCOM particles in the same density sucrose gradient fraction [43,61,62] or directly by visualization of protein and ISCOM particles together by immunoelectron microscopy using gold labelled antibodies to the protein.

4.2. Incorporation of non-amphipathic molecules into ISCOMs

While it is possible to insert or at least associate non-amphipathic molecules with ISCOMs, structural modifications such as partial denaturation of proteins using agents such as urea and mercaptoethanol [63], exposure to low pH [64–66] or high temperature [61] are needed to uncover some of the hydrophobic regions within proteins. For example, exposure to pH 2.5 buffers resulted in 15% and 14% incorporation of purified HIV-1 gp120 [64] and BSA [65] respectively into ISCOMs while 70°C heat treatment of ovalbumin (OVA) [66] or BSA [61] increased incorporation. These methods may lead to a loss of conformation dependent B cell epitopes, so an alternative strategy is the covalent attachment of fatty acids to soluble proteins. A number of soluble proteins, such as OVA, cytochrome C, Tamm–Horsfall glycoprotein and recombinant HIV-1 gp120, have been successfully incorporated into ISCOMs following attachment of palmitic acid through the ϵ -amino groups on lysine [67–70]. Mowat et al. [67] found palmitified OVA-ISCOMs were capable of inducing Delayed Type Hypersensitivity (DTH) responses in mice when injected into the footpad, unlike OVA, palmitified OVA, or palmitified OVA given with Quil

A or ISCOM matrix. Similarly, the addition of palmitic acid [71] or myristic acid [54] fatty acid tails during peptide synthesis allows the incorporation of peptides into ISCOMs. Peptides from 10–40 residues have been successfully modified with lipid tails and incorporated into ISCOMs, however there may be some size limitations on particular peptide sequences as some short, poorly-charged peptides may become too hydrophobic and difficult to incorporate into ISCOMs [12].

Other methods used to associate molecules with ISCOMs include chemically coupling peptides [72] and possibly proteins on to pre-formed ISCOM matrix or ISCOMs containing influenza envelope proteins [72–75]. Theoretically, coupling could be done through amine groups if phosphatidylethanolamine has been used as the phospholipid [12]. Weiss et al. [76] chemically coupled influenza nucleoprotein to *E. coli* lipopolysaccharide (LPS), by periodate oxidation, which then allowed association of 10–20% of the nucleoprotein with ISCOMs. The problem of incorporating non-amphipathic molecules into ISCOMs has also been approached by utilising genetic manipulation to re-engineer the protein to contain a transmembrane sequence. In one example of this [77], a protein was modified to retain a transmembrane region and this protein induced higher antibody responses in cats when incorporated into ISCOMs than did the native protein given alone or when combined with Quil A.

5. Other saponin based vaccine formulations

5.1. Liposomes

As an alternative to entrapping saponins into the structure of an ISCOM, Lipford et al. [78] re-hydrated liposomes in the presence of OVA peptides and Quil A, leading they believed, to the entrapment of the saponins into the internal space of the liposome. The resulting Quil A liposomes were effective at inducing an MHC Class I restricted CD8⁺ cytotoxic T lymphocyte (CTL) response to peptide pulsed or transfected target cells following a single dose in mice. Liposomes without Quil A failed to generate any CTL while simply mixing empty liposomes, peptide and Quil A induced a

lower CTL response [78]. The authors claim that the advantage of this system compared, with ISCOMs, is that there was no need to add lipid tails to the peptides, or if proteins were used, no denaturation would be necessary [78]. A lysteriolysin peptide from *Listeria monocytogenes* formulated in Quil A liposomes was also shown to promote specific CTL responses and enhance survival in mice challenged with 10 LD₅₀ of live *L. monocytogenes* organisms [79]. Interestingly Newman et al. [80] had earlier reported the ability of a purified saponin (QS-21) to induce OVA specific CTL in the absence of liposomes or other additives.

5.2. Microspheres

Considerable interest has been shown in the use of biodegradable microspheres for slow release or single injection vaccines [81–85]. As with other vaccine delivery systems, there is often the need to add an adjuvant to achieve the desired level of immune response when using microspheres. This was described by Cox et al. [84] using microspheres containing antigen and either Quil A or aluminium salts and subsequently microencapsulation of ISCOMs [85]. In studies by Cleland et al. [81], recombinant gp120 from the HIV-1_{MAN} strain, was microencapsulated into polylactide co-glycolide (PLG) microspheres which induced long lasting antibody responses in guinea pigs after two subcutaneous (SC) doses. This response was enhanced five fold by the addition of 50 µg of QS-21. These studies have been extended to baboons where high antibody titres and virus neutralization titres were induced using microspheres containing QS-21 [83]. Thus, saponins and ISCOMs can be used to increase the potency of microencapsulated antigens.

5.3. Aluminium salts

The addition of saponins was one of the early methods used to improve the responses obtained with aluminium (alum, Al(OH)₃ or Al-PO₄) adjuvanted vaccines. The potency of bacterial vaccines to *Bacterioides nodosus* [86] and *Corynebacterium pseudotuberculosis* [87] in sheep, *E. coli* in cattle [88] and *Bordetella bronchiseptica* in mice [89] have all been increased when Quil A or saponin was

added to the aluminium precipitated vaccine. QS-21 has also been shown to increase responses in mice to an aluminium precipitated peptide-conjugate vaccine although some studies have found that the aluminium gave no increase in response over the use of QS-21 alone [90] or at best a small increase [91]. Rarely does the addition of saponin to aluminium precipitated proteins cause a lower response and only isolated examples have been reported [92]. In some instances the aluminium salt is used to stabilise the antigen rather than for any additional adjuvant activity [93]. Very few studies have looked at combining ISCOMs with aluminium precipitated proteins, however in one study in mice [21] using AlPO₄ and ISCOMs containing a gonococcal pore protein, a good antibody response was obtained but no comparisons were made with ISCOMs without AlPO₄.

Recent work by So et al. [23] led to the identification of a component in crude *Quillaia saponaria* saponin preparations, termed QS-L1, that was found to have adjuvant activity in mice but only when used with alum adsorbed recombinant HBV Surface Antigen (rHBsAg). No adjuvant activity was detected when QS-L1 was added to soluble rHBsAg unlike previous studies with QS-7, 17, 18 or 21 which were adjuvant active in formulations with soluble proteins [19]. This observation possibly reflects the unique properties of this fraction as well as variation associated with different protein antigens.

6. Mode of action

Adjuvants may act in several ways. These include the formation of a depot from which antigen is slowly released, efficient targeting of the antigen to lymphoid organs and antigen presenting cells (APC) and modulation of the properties of induced immune responses [94]. Although the adjuvant activities of saponins and ISCOMs are not yet completely understood, an increasing number of reports have recently described various aspects of the initiation, development and modulation of immune responses after immunization with various saponins such as QS-21 or ISCOMs. Due to the relatively recent identification and limited availability of QS-21 much of the

published work in this area has been done with ISCOMs, Quil A or crude saponins.

6.1. Uptake and distribution

Early studies by Chedid et al. [95] showed that ^{125}I -KLH when given into the footpad of mice along with crude *Quillaja* saponin resulted in increased radioactivity at the site of injection and the spleen compared to ^{125}I -KLH alone. However, retention of antigen at the site of injection was also demonstrated with other compounds that were not adjuvant active. The uptake and organ distribution of ISCOMs after intraperitoneal (IP) and SC injections has also been analysed using influenza-ISCOMs containing radioactively-labelled influenza virus envelope glycoproteins [96,97]. The clearance and organ distribution of influenza-ISCOMs was rapid after administration by either of these two routes with less than 5% of the injected radioactivity recovered in tissues examined 3–12 h after ISCOM administration [96,97]. Although, following IP dosing with influenza-ISCOMs, the recovery of radioactivity was significantly greater in spleens from ISCOM-treated mice compared to mice given influenza micelles (flu-m) [97]. Influenza-ISCOMs injected SC were distributed to the lymph nodes with higher efficiency than flu-m alone or when flu-m were mixed with ISCOM matrix [98]. Administration of influenza-ISCOMs also appeared to increase the retention of antigen in lymphoid organs [97,98] compared to flu-m or flu-m mixed with ISCOM matrix, which suggests that ISCOMs are efficiently targeted to lymphoid organs where they may persist for a substantial time. These results also demonstrate that retention of antigen by depot formation is not a component of the adjuvant activity of ISCOMs [96–98].

Consistent with the proposed efficient targeting of ISCOMs to the draining lymph nodes after SC immunization, both B and T cell responses are initiated in these organs following a single SC injection [96,99]. These responses were rapid and transient, with peak activities from day 5 to 8, whereas the parallel responses in the spleen developed more slowly but were of a longer duration. One explanation for the difference in distribution between ISCOMs and flu-m may be that ISCOMs

are taken up more efficiently by APC in the spleen and lymph nodes. Support for this possibility comes from electron microscopy studies on the uptake of influenza-ISCOMs or flu-m by peritoneal lavage cells. Just 30 seconds after IP injection, ISCOMs were seen adhered to macrophage cell membranes or contained within phagolysosomes [100] while attachment of flu-m was rarely observed.

In a recent study, lipophilic fluorescent carbocyanine dyes were used to follow the cellular distribution of inactivated rabies virus antigen and ISCOMs containing rabies virus antigen [101]. Following IP or intravenous injection, both rabies antigen and rabies ISCOMs were taken up by splenic macrophages. The rabies antigen primarily localized to marginal zone macrophages (MZM) and to some extent also to red pulp macrophages. MZM are important for the uptake of particulate antigens and have been shown to play a role in the removal and elimination of antigens. Rabies-ISCOMs were also taken up by MZM but preferentially localized to marginal metallophilic macrophages. This population of macrophages, believed to be involved in antigen processing [102], is located on the border of the marginal zone, close to the major site of antibody production in the spleen [101]. This difference in cellular distribution may explain why rabies-ISCOMs, but not rabies antigen, efficiently induced immune responses at low doses [101]. In conclusion, these studies suggest that increased localisation to lymphoid organs together with effects on the cellular uptake of antigens may contribute to the adjuvant activity of ISCOMs and possibly other saponin containing formulations.

6.2. Antigen presentation

Information obtained from in vitro studies has demonstrated that ISCOMs influence the functional properties of APC in several ways. In a study by Villacres-Eriksson et al. [103], incubation in vitro with influenza-ISCOMs or ISCOM matrix prepared from Quil A stimulated spleen cells to produce both membrane-associated and soluble IL-1. In contrast, only membrane-associated IL-1 was produced after incubation with flu-m [103]. Stimulation of fractionated cell populations with influenza-ISCOMs or ISCOM matrix alone demonstrated that soluble IL-1

was produced by adherent but not by non-adherent spleen cells. In a related study [28], *Quillaia* saponins in free form or incorporated into ISCOM matrix or influenza-ISCOMs stimulated IL-1 secretion by murine peritoneal cells in vitro. Stimulation with influenza-ISCOMs prepared from QH-703 (ISCOPREP™703) resulted in levels of soluble IL-1 comparable to those induced by incubation with *E. coli* lipopolysaccharide (LPS) [28]. Taken together, these results demonstrate that ISCOMs, ISCOM matrix and purified saponins are able to activate APC to produce IL-1. The finding that IL-1 was produced by adherent cells [103] and peritoneal cells [28] suggests that macrophages are involved, consistent with the role of these cells in the uptake of ISCOMs after injection [100,101]. The capacity to stimulate IL-1 production may therefore contribute to the adjuvant effects of ISCOMs. However, the role of IL-1 in the development of immune responses is not clearly understood and a correlation between IL-1 production in vitro and the induction of antibody and cellular immune responses in vivo remains to be established.

ISCOMs have also been reported to upregulate the expression of MHC class II molecules on APC. Thus, the frequency of peritoneal cells expressing MHC class II was significantly higher in mice immunized with influenza-ISCOMs than in mice injected with ISCOM matrix or flu-m [100]. This finding was extended by Bergström-Mollnaglu et al. [104] who showed that antigen stimulation in vitro of spleen cells primed with influenza-ISCOMs or HIV gp160-ISCOMs increased the frequency of cells expressing MHC class II in an antigen dose-specific manner. The increased MHC class II expression was at least partly dependent on the antigen-specific production of IFN- γ because a monoclonal antibody to IFN- γ reduced the proportion of MHC class II positive cells by 50% [104].

A critical issue with respect to adjuvant activity is to what extent distinct APC populations contribute to the presentation of antigens. This question was addressed in a recent report where naive splenic dendritic cells (DC) and B cells and peritoneal cells were pulsed in vitro with influenza-ISCOMs and used to stimulate T cells primed with influenza-ISCOMs [105]. All of these populations of APC stimulated T cells to proliferate and produce cyto-

kines. DC and B cells were the most efficient with respect to proliferation and secretion of IL-2, whereas only DC induced high levels of IFN- γ . From these results it appears that several populations of APC are able to take up and present ISCOM borne antigens. The efficient induction of IFN- γ production by DC is of particular interest because this population of APC has been shown to be important producers of IL-12, a cytokine which is required for effective generation of cellular immune responses [106]. Immunization with influenza-ISCOMs was recently demonstrated to induce production of IL-12 at levels high enough to be detected in the serum of injected mice [107].

Little work has been done with QS-21 in this area although one interesting study examined whether QS-21 micelles or monomers were the active forms of the molecule [90]. When QS-21 was given to mice at concentrations well below the CMC of QS-21 (26 mM) it was still adjuvant active suggesting that the monomer is active and can presumably act on APC [90]. Wu et al. [108] assessed the actions of QS-21 on different APC by using particulate silica or carageen to paralyse phagocytic APC. Following this treatment they were then unable to induce specific CD8⁺ CTL responses in mice vaccinated with QS-21 and OVA but antibody responses or antigen-specific lymphocyte proliferation were not effected [108]. These studies, and other in vitro studies, suggest a critical role for macrophages for QS-21-mediated CD8⁺ CTL responses but a lesser role in promoting antibody responses.

6.3. Cytokine induction

Regulatory Th cells are essential for the development of antibody and CTL responses to foreign antigens. With respect to their cytokine production after activation, murine Th cells can be divided into at least two functionally distinct sub-populations. Th1 cells produce the cytokines IL-2 and IFN- γ and mediate certain antibody-independent immune responses as well as promoting certain antibody responses. Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and are considered to provide help essential for antibody production [109]. The importance of the Th cell subsets for generation of protective immunity has been demonstrated in several experimental models and human diseases and suggests that vacci-

nation against certain infectious diseases may be dependent on efficient means to induce T cell responses with desired properties [110-112]. The use of adjuvants with distinct immunomodulatory properties represents one approach to achieve this aim.

The ability of saponin based formulations to induce strong Th cell responses is well established. Studies in sheep [113] demonstrated that high levels of IFN- γ was present in the lymph following injection of Quil A in the presence or absence of antigen; replacing the Quil A with Al(OH) $_3$ did not induce a similar response. A number of studies have examined the development of Th1-like and Th2-like T cell responses after injection of saponin containing vaccines, particularly ISCOMs and QS-21. Activation of T cells by ISCOMs was first described by Fossum et al. [114], who reported that immunization with influenza-ISCOMs induced spleen cells which proliferated and secreted IL-2 after antigen stimulation in vitro. These observations were extended in a study [115] which showed that the production of IL-2 and IFN- γ by spleen cells primed with ISCOMs was dependent on CD4 $^{+}$ T cells. The ability of ISCOMs to induce T cells producing IL-2 and IFN- γ has since been demonstrated for a number of antigens (Table 2) and it is now well established that ISCOMs strongly promote the development of Th1 type T cell responses. Additional support for this observation is that ISCOMs elicit high levels of antibodies of the IgG2a subclass (Table 2). A shift to

production of antibodies of this subclass is dependent on IFN- γ , whereas IL-4 is important for the generation of high levels of IgG1 [116]. The relative production of these IgG subclasses in mice can therefore be used as a surrogate marker for the generation of immune responses of a Th1 or Th2 type.

The generation of Th2 responses after ISCOM immunization is less clear cut and appears to vary with the antigen used, the choice of cytokines analysed and the type of cytokine assay. The production of IL-4 by T cells primed with ISCOMs has been reported to be low or undetectable when determined as the cytokine concentration in cell culture supernatants (Table 2). However, IL-4 may be rapidly consumed [117,118] and therefore, the low levels of IL-4 in culture supernates may not therefore reflect the true responses in vivo. In support of this, immunization with ISCOMs containing an antigen (PSA-2) from the parasite *Leishmania major* induced high numbers of T cells producing IL-4 as detected in an ELISPOT assay but only trace amounts of IL-4 were detected in parallel cell culture supernates [119]. Moreover, vaccination of C3H/He mice with PSA-2-ISCOMs did not protect them against *L. major* infection despite the activation of high numbers of T cells secreting IFN- γ . As protection against *L. major* depends on the generation of Th1-like T cells producing IFN- γ [111,120] and susceptibility correlates with the presence of IL-4 [121-123], these findings suggest that

Table 2
Cytokine pattern and IgG subclass profile of immune responses induced by *Quilbina* saponin adjuvants

Antigen	Adjuvant	<i>Quilbina</i> saponins	IL-2	IL-4	IL-5	IL-10	IFN- γ	IgG1	IgG2a	Reference
Influenza	ISCOMs	Quil A	+	ND ^a	ND	ND	ND	ND	ND	[113]
Influenza	ISCOMs	Quil A	+	ND	ND	ND	+	+	+	[113]
Ovalbumin	ISCOMs	Quil A	+	-	+	ND	+	+	+	[125]
Influenza	ISCOMs	Quil A	+	-	ND	+	+	ND	ND	[108]
Influenza	ISCOMs ISCOM matrix	Quil A	+	±	ND	ND	+	+	+	[98]
EBV gp340	ISCOMs ISCOM matrix	Quil A, Iscoprep703	+	-	ND	±	+	+	+	[126]
Ovalbumin	ISCOMs	Iscoprep703	+	+	ND	±	+	+	+	[124]
Influenza	ISCOMs	Quil A	+	+	ND	ND	+	ND	ND	[96]
<i>L. major</i> PSA-2	ISCOMs	Iscoprep703	ND	+	+	ND	+	+	+	[119]
Influenza	Free form	QH-C	+	± ^c	+	± ^c	+	+	-	[127]
Ovalbumin	Free form	QS-21	+	+	+	ND	+	+	+	[42]

^a Not determined.

^b As determined by ELISPOT.

^c Cytokine levels determined in serum.

the activation of Th2-like T cells by ISCOM vaccination was sufficient to abrogate the protective Th1 effects. In addition, the induction of IL-4 by OVA in ISCOMs has been reported to be comparable to that of OVA in $Al(OH)_3$, [124] an adjuvant with extremely high capacity to induce Th2 responses. The strong ability to increase IgG1 responses to antigens provides further support for the involvement of IL-4 in immune responses to ISCOMs (Table 2). ISCOMs have also been reported to induce production of IL-5 and IL-10 (Table 2). Immunization with OVA-ISCOMs or PSA-2-ISCOMs generated T cells producing significant amounts of IL-5 [119,125]. The effects of ISCOMs on IL-10 production are unclear as both an increase and decrease in production of IL-10 have been reported. Recent results suggest that *Quillaia* saponins may down-regulate the production of IL-10 in a dose-dependent way [105,124,126]. It can be concluded that ISCOMs, in most cases, function as a potentiator of a Th1 type immune response but are also able to induce a concomitant Th2 response.

When assessing the contribution of Th2 T cells it is important to consider several variables such as which Th2 cytokines were analysed, the assays used to measure cytokine production, the immunogenic and immunomodulatory properties of the antigen, the mouse strain used, the route of administration, the dose levels of antigen and the dose, source and purity of the saponin. On this last point, most studies to date with ISCOMs have used Quil A as a source of saponin. The recent identification and isolation of defined fractions of *Quillaia* saponins has raised the question whether ISCOMs containing such components retain the adjuvant activities displayed by ISCOMs made from Quil A. Analysis of immune responses to OVA-ISCOMs prepared from ISCOPREP™703 suggests that these activities are consistent with previous studies using Quil A (Table 2) [124]. Moreover, immunization with influenza vaccine combined with QH-C induced both Th1 and Th2 cytokines (Table 2) [127]. Although this *Quillaia* saponin fraction elicited strong serum antibody responses and high serum levels of IFN- γ , antigen-specific IgG2a was not detected. In contrast, the saponin adjuvant QS-21, has been reported to augment the production of IgG2a and IgG2b antibodies as well as some IgG1 [19]. Analysis of T cell

responses to OVA formulated with QS-21 showed induction of cytokines associated with both Th1 and Th2 cells. Thus, spleen cells primed with OVA formulated with QS-21 efficiently secreted IL-2 and IFN- γ after antigen stimulation in vitro and produced levels of IL-4 and IL-5 which were comparable to those induced by antigen plus $Al(OH)_3$ (Table 2) [42].

The importance of the immunomodulatory properties of the antigen was supported in a recent study with the attachment (G) and fusion (F) proteins of respiratory syncytial virus (RSV) and QS-21 [128]. Immunization with the G protein resulted in responses from T cells isolated from the bronchoalveolar lavage fluids with Th2-like properties whereas the F protein generated responses that were more Th1 in character [128]. The authors concluded that the primary effect of the QS-21 in this experimental model was amplification, not modulation of the immune response. Clearly, further studies are required to delineate the adjuvant activities of the purified *Quillaia* saponins in more detail.

6.4. CTL induction

The induction of CTL responses generally requires that antigens are processed in the cell cytosol to generate peptides which are presented at the cell surface in the context of MHC class I molecules. Exogenous antigens must therefore be able to enter the cytosol to give rise to peptides which can be presented to MHC class I restricted CTL [129]. Adjuvants can be useful for CTL induction by facilitating this process. One way to achieve this is for the adjuvant to interact with the cell membranes so that antigen together with the adjuvant is deposited into the cytosol. The adjuvant can also induce the production of Th1-like cytokines which are necessary for the development of these cellular immune responses [11,130].

ISCOMs have been demonstrated to induce CD8⁺ MHC class I restricted CTL to a number of antigens after immunization by several different routes of administration. This was first illustrated with recombinant HIV-1 gp160-ISCOMs and influenza-ISCOMs [131]. ISCOMs have also been used to induce CTL to measles virus F-protein [132], a heat shock protein of mycobacteria [133], OVA [66,67], E6 and E7

proteins of HPV [134], SIV proteins [135] and RSV [136]. Intranasal administration of influenza-ISCOMs, RSV-ISCOMs and immunization by oral administration of palmitified OVA-ISCOMs also result in the generation of CTL [137–139]. Soluble QS-21 is also a potent inducer of CTL responses in mice, which has been demonstrated using OVA [80], recombinant HIV-1 gp160 [140], compound peptides of the HIV-1 gp160 immunodominant CTL epitope [141], a recombinant human CMV glycoprotein B [142], the F protein of RSV [143] and ras expressing tumour cells [144]. QS-21 was also used with SIV proteins to induce CTL in nonhuman primates [145]. Other adjuvants have also been shown to induce CTL's under certain circumstances. These include oil-in-water emulsions formulated with OVA [146] or HIV-1 gp120 [146], Al(OH)_3 with influenza [147], OVA with MPL-liposomes [148] and lipopeptides [149] but these examples are few compared to those of the saponin based adjuvants.

The mechanism by which ISCOMs and QS-21 induce CTL responses is not clear, but it is likely that these adjuvants associate with antigen and facilitate entry into the cell cytoplasm. Due to their surface-active properties [11], it is possible that the *Quillaja* saponins play a role in this process by intercalating with cholesterol in the cell membrane to form pores, which have been observed in electron micrographs [46], through which the saponin and antigen could pass into the cytoplasm. Supporting this mechanism is the finding that ISCOMs containing the measles virus F-protein have been reported to sensitize target cells in vitro for lysis by CD8^+ MHC class I-restricted CTL clones [132]. When a cell line which had lost the ability to generate peptides presented by MHC class I molecules was used as the APC, no lysis was detected, demonstrating that processing in the cytosol of measles F protein contained in the ISCOMs was necessary. The authors concluded that the ISCOMs might incorporate into cell or endosomal membranes, thereby exposing the incorporated antigen to cytosolic proteases [132].

7. Toxicity

The issue of toxicity with saponin based adjuvants has been raised at various times as a potential

impediment to their widespread use in human and veterinary vaccines. Orally administered crude saponins are relatively benign and appear to only effect weight gain in mice and rats [150,151] and this effect could be reversed in rats by the addition of cholesterol to the diet [151]. However, saponins are toxic at high doses when administered parenterally. The LD_{50} for intravenously administered Quil A is 0.67 mg/kg in rats [152] and doses of greater than 25 μg of Quil A given IP can be toxic in the $\text{B}_6\text{D}_2\text{F}_1$ strain of mice [153]. Lethality in other strains of mice has also been reported with both IP and SC doses of ISCOMs containing from 10–50 μg of Quil A [21,64,154]. When examined at autopsy, one study showed saponin-treated mice to have liver degeneration [21]. Despite these findings with Quil A and Quil A-ISCOMs in rodents there has been little toxicity with parenterally administered Quil A derived ISCOMs or QS-21 in larger animals such as rhesus monkeys, horses, cattle, sheep, chickens, dogs or cats [64,155–159] or with free Quil A in cattle and sheep [113,160], although there was some toxicity reported with free Quil A in cats [20]. Local toxicity was investigated in rats following the injection of a measles-ISCOM vaccine made with Quil A saponin or with Quil A into the gastrocnemius muscle [161]. Histopathological examination showed a lower inflammatory response to the vaccine when the Quil A saponins were incorporated into ISCOMs compared to free Quil A [161]. This is unlikely to be due to selective incorporation of certain saponin fractions from Quil A into ISCOMs as other studies have shown there is no selective incorporation of particular saponins into ISCOMs [21].

The concerns over toxicity were a major stimulus to fractionate crude saponins and Quil A. Kensil [19] investigated the major saponin peaks (QS-7, 18 and 21) for lethality in mice, hemolysis of SRBC and the levels of IgE antibodies induced. Lethality of the saponin fractions was assessed in CD-1 mice given graded doses of up to 500 μg of saponin intradermally [19]. This showed that QS-18 was quite toxic, even more so than whole Quil A, while QS-21 had low toxicity and QS-7 showed no lethality [19]. Other fractions were identified that were toxic for mice but were well tolerated in kittens [20]. Increasing hemolytic activity was seen with fractions QS-17, QS-18 and QS-21 respectively but QS-7 was not hemolytic at levels up to 200 $\mu\text{g}/\text{ml}$ [19]. They

concluded that there was no correlation between hemolytic activity, lethality and adjuvant activity [19]. No specific IgE was induced with any of these four fractions, which contrasts with other studies of Quil A [162], *Gypsophylla* saponins [163] or saponins from un-named sources [164,165].

Rönnberg et al. [27] examined the lethality, cytotoxic and hemolytic properties of QH-A, QH-B, QH-C and ISCOM matrix formed with various ratios of these fractions. Lethality studies in ICR mice showed low levels of toxicity associated with QH-A (0/10 deaths at 400 µg) and QH-C (0/10 deaths at 400 µg) or ISCOM matrix made from QH-C (0/10 deaths at 800 µg) but there was toxicity associated with the use of QH-B (7/10 deaths at 400 µg) [27]. Cell cytotoxicity and hemolysis were also significantly reduced when QH-C or various ratios of the 3 fractions were incorporated into ISCOM matrix compared to free QH-A, QH-B or QH-C [27]. Absence of toxicity was found in studies with free QS-L1, with no deaths following intradermal dosing of CD-1 mice with 500 µg of QS-L1 and no hemolysis of SRBC at concentrations of up to 125 µg/ml of QS-L1 [23].

Some purified saponins, like QS-21, ISCOMPREP™703, and formulations which utilise these saponins, such as ISCOM matrix and influenza-ISCOMs, have been tested for systemic and local toxicity in rats and rabbits as part of their pre-clinical evaluation as well as mutagenicity and pyrogenicity. In rabbits 200 µg of QS-21 given intramuscularly was well tolerated with little evidence of toxicity [42] and similar results were obtained with ISCOMPREP™703, ISCOM matrix and influenza-ISCOMs in rats and rabbits [166]. The encouraging data generated from these studies has led to safety and efficacy trials being conducted in human volunteers. Some of the other fractions such as QS-7 and QS-L1 have yet to be fully evaluated for toxicity.

8. Studies of saponin based vaccine formulations in animal models

A comprehensive review of all of the animal studies using saponin based adjuvants is beyond the scope of this review; several excellent reviews are recommended [9,11,15,56,167]. A wide range of

animal species including rodents, guinea pigs, rabbits, pigs, cats, dogs, cattle, sheep, monkeys and baboons have been used to test Quil A, QS-21 and ISCOMs with an emphasis on vaccines directed at viruses and parasites rather than bacteria. These vaccines have generally been based on proteins or glycoproteins either derived from the organism or produced recombinantly [9,41,56,77]. Quil A and QS-21 have also been used to adjuvant non-protein antigens such as polysaccharides and lipids, which are generally considered to be T-independent antigens. Most non-saponin based adjuvants fail to enhance immune responses to these types of antigens, however Quil A was shown to have some adjuvant capacity with certain antigens such as dextran sulphate [168], TNP-LPS or TNP-Ficoll [153,169] and QS-21 with *E. coli* polysaccharide [170,171] and detoxified *E. coli* LPS [171]. This effect was not universal as addition of QS-21 to capsular polysaccharides from *Staphylococcus aureus* failed to enhance antibody levels unless the polysaccharides were conjugated to protein carriers [172].

When studies have compared a range of adjuvants in experimental animals [41,154,173–177] various rankings have been obtained. These range from QS-21 or ISCOMs being superior to 'gold standards' like Freund's adjuvant [19,42,64,173,177] to being equivalent with other adjuvants [174,176] or in some cases inferior to other adjuvants [154,175]. These results should be considered cautiously as much depends on the dose of antigen and adjuvant, the animal species and the criteria on which the assessment is being made. Few studies have extensively evaluated all of the saponin adjuvants or even QS-21 or ISCOMs against a panel of adjuvants using a number of antigens, species, doses and most importantly, equivalent read-out systems.

Intramuscular (IM) and SC routes have commonly been used for saponin based vaccines with few studies using the oral or intranasal routes for vaccination. Ben Ahmed et al. [178] reported no protection from a homologous influenza challenge in mice given a single low level dose of influenza-ISCOMs, 0.25 µg hemagglutinin (HA), administered intranasally. This is consistent with earlier reports of intranasal ISCOM vaccination where at least two doses of 1 µg HA [179] were required for protection against influenza and 3 doses of 3.5 µg of RSV

protein were required for protection against an RSV challenge [136]. In intranasal studies using BALB/c in our laboratories, good serum antibody responses and protection were only seen with two high doses of influenza-ISCOMs, consisting of 5 µg HA and 10 µg of either Quil A or ISCOPREP™703 (Table 3). These experiments were carried out in non-anaesthetised mice as anaesthetic tends to enhance responses even with small doses of vaccine, possibly due to uptake via the lungs. Further studies have also shown priming of peptide specific CTL cells after the application of ISCOMs intranasally [139].

Results obtained with oral vaccination using ISCOM vaccines have been variable, with partial protection/clearance reported in some systems [180–182] and none in others [183]. No studies have been reported yet on the use of QS-21 in oral or intranasal vaccines although there are reports of the successful use of a modified (deacylated) form of QS-21 for the intranasal delivery of insulin [36,184]. There is some attraction to the oral route if saponins can be used effectively because of the very low toxicity of orally applied saponins [11,150,151]. However the local and systemic toxicity surrounding the use of saponins intranasally has yet to be fully determined. Results for IM and SC administration have generally been more consistent and all animal and human

vaccines currently being developed utilise these routes of administration.

ISCOM based vaccines also have the ability to induce CTL which may be important in the protection/recovery from some viral infections and in immunotherapy. The induction of CTL against specific antigens has been demonstrated for a number of ISCOM based vaccines in both mice and non-human primates. The ability of ISCOMs and QS-21 to induce CTL's seems to be more potent, compared to other adjuvants [9,140]. Unfortunately, only a few studies have been completed comparing the relative potency with respect to CTL induction and it is not known whether any of these results can be reproduced in humans.

9. Vaccination in the presence of maternal antibodies

An area of concern in some developing countries is that vaccination is required very early in life. The failure of vaccines to protect babies can often be linked to high levels of maternal antibodies, as has been seen with measles vaccines [185]. To determine if a vaccine utilizing ISCOMs could overcome this problem van Binnendijk *et al.* [186] looked at the

Table 3
Intranasal vaccination of unanaesthetised mice with A/PR8/34 influenza-ISCOMs

Gp	Treatment ^a	HA dose µg ^b	Serum IgG GMT ^c	Serum IgA GMT ^c	Following live influenza challenge ^d	
					Weight change ^e	Survivors ^f
1	Flu-ISCOMs	5	230	174	2.2	93
2	Flu	5	177	< 100	-22.4	40
3	Flu-ISCOMs	1	131	107	-25.9	10
4	Flu	1	< 100	< 100	22.8	0
5	PBS	0	< 100	< 100	-22.6	0
6	Flu-ISCOMs ^g	0.5	6961	< 100	-0.2	100
		p < 0.05	gp1 > 2.3,4,5	N.S	gp 1 < 2.3,4,5	gp 1 > 5

^a Female BALB/c mice 8 weeks old were administered either influenza-ISCOMs, or purified influenza protein derived from egg grown A/PR8/34 influenza. Unanaesthetised mice were given 12 µl onto the nares of the nose using an autopipette and allowed to inhale the formulation naturally. Intranasal dosing was carried out at 0 and 21 days, mice were bled 14 days later and challenged with live A/PR8/34 influenza virus. ISCOMs were made with ISCOPREP™703 saponin.

^b Influenza HA was estimated to be approximately one third of the total influenza protein.

^c Geometric mean titre calculated by ELISA using plates coated with A/PR8/34 whole lysate.

^d Mice were challenged with approximately 1×10^6 TCID₅₀ of live homologous A/PR8/34 influenza virus and monitored for clinical symptoms, daily weight loss and death. Weight change is the mean of the weight changes from the day of challenge to day 5 post challenge.

^e This group received the Flu-Iscoms subcutaneously as a single dose at day 0.

response using an ISCOM based vaccine containing measles hemagglutinin and fusion proteins. They compared this vaccine with a live attenuated measles vaccine and a recombinant vaccinia virus expressing the same proteins in macaque monkeys in the presence or absence of passively transferred measles-specific antibodies. As little as 0.1 IU of virus-neutralizing antibody/ml of serum abrogated the induction of virus neutralizing antibodies in monkeys vaccinated with the attenuated measles vaccine or with vaccinia virus expressing the measles proteins whereas vaccination with measles-ISCOMs elicited high titres of such antibodies [186].

Studies in horses have demonstrated that ISCOMs containing envelope antigen from Equine Herpes 2 virus elicited virus neutralizing antibodies in two week old foals in the presence of maternal antibodies and the ISCOM vaccine induced protective immunity against disease whereas conventional vaccines were unsuccessful [187]. These results suggest that administration of antigen in ISCOMs can override the negative effects of maternal antibodies on vaccination efficacy. However, the mechanisms by which this is achieved remains to be clarified.

10. Veterinary vaccines using saponin based formulations

While there are a number of commercial veterinary vaccines which utilise Quil A or crude saponins, there are only two licensed vaccines that utilise ISCOMs or QS-21 with another two undergoing evaluation. Brief details of these newer saponin based vaccines are outlined below.

10.1. ISCOMVAC FLU vetTM

The only licensed veterinary ISCOM-based vaccine is used to protect horses from equine influenza and has been produced by Iscotec (Sweden) and Mallinckrodt (UK) since 1989. It contains two strains of inactivated equine influenza virus and 300 µg of Quil A [9]. From the time the product was launched, over 1 million doses of the vaccine have been sold in Sweden with *no* reported adverse side effects.

10.2. QuilvaxTM programs

This range of products is being developed by Aquila Biopharmaceuticals. Quilvax-FelVTM is given to cats to prevent feline leukemia and was launched in 1990/91 [157,188,189]. It contains recombinant FeLV gp70 antigen absorbed to Al(OH₃) and is supplemented with 20 µg QS-21. This product is available in the US and Europe. Quilvax-LTM has received marketing approval for the prevention of canine Lyme disease. It contains recombinant OspA and OspB, the major outer surface proteins of *Borrelia burgdorferi*, formulated with 50 µg QS-21 [158,190]. Quilvax-MTM is being developed for the prevention of bovine mastitis. It contains *E. coli* antigen and fibronectin binding protein, derived from *S. aureus*, antibodies to which prevent bacterial adhesion *in vivo*.

11. Clinical testing

Clinical trials have been completed using both ISCOMs and ISCOM matrix containing purified saponins (ISCOPREPTM703) and the highly purified saponin QS-21. At this stage only safety data on the local and systemic effects of ISCOM matrix in human volunteers has been published [166]. At doses of 25 to 200 µg, ISCOM matrix caused a mild ache/pain at the site of injection, with onset at 1–24 h and duration of 4–48 h. The effect was not strictly correlated with dose as 4/6 patients receiving 200 µg and 6/6 patients receiving 100 µg reported pain whereas 4/8 volunteers reported a similar effect with placebo [166]. There was only occasional incidence of headache, pyrexia or drowsiness for volunteers given ISCOM matrix and this occurred at a similar frequency in the placebo group [166]. Phase I Clinical trials with influenza-ISCOMs and influenza combined with ISCOM matrix are currently underway.

Clinical trials with QS-21 in a melanoma immunotherapeutic GM2 ganglioside-KLH vaccine were used to demonstrate enhanced and prolonged IgG and IgM antibody titres to the GM2 [191–193]. This product is now in Phase III trials. Doses of the GM2 ganglioside coupled to KLH when given with \leq 100 µg of QS-21 SC induced mild local tenderness and

inflammation at the dose site which lasted for 2 to 4 days with brief but low grade fever and malaise with no significant incapacitation [192]. A 200 µg dose QS-21 plus vaccine caused unacceptable local and systemic effects including fever and malaise in 30% of the vaccinees with visible local reactions and discomfort in the injected extremity for 5-10 days. The 100 µg dose of QS-21 was determined as the optimal dose with this vaccine [192]. Further clinical trials of QS-21 with HIV-1, HSV and influenza are in progress [11]. Interestingly there appear to be plans to test other purified saponins such as QS-7 [11], the saponin fraction with the lowest lethal toxicity from the four most abundant saponins purified from crude saponin [19].

12. Conclusions

The approach of using saponin based vaccine delivery systems has been considerably refined since the first trials in the 1950s. When one examines the specifications for QS-21, purity $\geq 98\%$ by RP-HPLC, identity by FAB-mass spectroscopy and infrared spectroscopy, residual moisture $\leq 5\%$, endotoxins ≤ 10 U/mg, residual solvents ≤ 50 ppm each; [42], some appreciation is gained for the efforts that have been invested into the purification of saponins and the increasing understanding of the mechanisms by which this novel class of adjuvant act. Some purified fraction(s) of *Quillaja saponin*, either free or incorporated into ISCOMs or ISCOM matrix may however, still be too reactogenic for use in humans with particular vaccines and other saponin species which appear to have lower reactivity, such as QS-7, may be needed. Alternatively, saponins with very low toxicity might be reserved for pediatric vaccines.

A clearer picture is being developed with respect to which saponins trigger specific immune cellular responses as the type of cytokines induced by saponins and ISCOMs are determined. While the classic Th1 cytokines IFN- γ and IL-2 are induced in abundance by vaccines containing QS-21 or ISCOMs, there is now clear evidence that Th2 cytokines are also induced with some proteins and this may have a significant effect on the level of protection obtained by vaccination against particular dis-

eases. Another critical finding for saponin based adjuvants will be whether QS-21, ISCOMs or ISCOM matrix, when combined with various antiviral and anti-cancer vaccines, are able to induce significant and sustained levels of active CTL in humans.

Several other questions remain to be resolved, such as whether there will be an immune response generated to these new vaccine components and what this effect might have on subsequent boosters or other vaccines using these components. The initial finding for QS-21 is that no antibody response is induced to QS-21 in human trials [192] or in animals to ISCOM components [114]. Other remaining questions include the levels of IgE generated with saponin based vaccines and the crucial question of longevity of the immune response that is generated. Thus, the promising area of saponin based human vaccines have entered a significant stage in their development with ongoing human clinical trials and the results over the next few years will determine their future applications in human medicine.

Acknowledgements

The authors wish to thank the following people who contributed to this article. Michael Kleinig for producing Fig. 1, Charles Quinn for work involved in Table 3, and Ian Gust and others for their helpful comments and contributions to this review.

References

- [1] I. Milgate, D.C.K. Roberts, The nutritional and biological significance of saponins, *Nutr. Res.* 15 (1995) 1223-1248.
- [2] S.W. Applebaum, S. Marco, W. Birk, Saponins as possible factors of resistance of legume seeds to attack of insects, *J. Agric. Food Chem.* 37 (1986) 1185.
- [3] D. Oakenfull, G.S. Sidhu, Toxicants of plant origin, in: P. Cheeke (Ed.) *Glycosides Vol. 11*, CRC Press, Boca Raton, 1989, pp. 97-141.
- [4] A.M. Rouhi, Researchers unlocking potential of diverse, widely distributed saponins, *C&EN* September 11 (1995) 28-35.
- [5] R.G. Espinet, Nuevo tipo de vacuna antiaftosa a complejo glucovirico, *Gac. Vet.* 74 (1951) 1-13.
- [6] K. Dalsgaard, Thin layer chromatographic fingerprinting of

- commercially available saponins, *Dnn. Tidskr. Farm.* 44 (1970) 327-331.
- [7] B. Morein, B. Sundquist, S. Höglund, K. Dalsgaard, A. Osterhaus, Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses, *Nature* 308 (1984) 457-460.
 - [8] G.F.A. Kersten, D.J.A. Crommelin, Liposomes and ISCOMS as vaccine formulations, *Biochim. Biophys. Acta* 1241 (1995) 117-138.
 - [9] I.G. Barr, G.F. Mitchell, ISCOMs (immunostimulating complexes): The first decade, *Immunol. Cell Biol.* 74 (1996) 8-25.
 - [10] M. Tomasi, T.L. Hearn, ISCOMs, liposomes, and oil based vaccine delivery systems, in: H. Kiyono, P.L. Ogra, J.R. McGhee (Eds.), *Mucosal vaccines*, Academic Press, San Diego, 1996, pp. 175-186.
 - [11] C.R. Kensil, Saponins as vaccine adjuvants, *Crit. Rev. Ther. Drug Carrier Syst.* 13 (1996) 1-55.
 - [12] B. Morein, K. Lövgren, B. Rönberg, A. Sjölander, M. Villacres-Eriksson, Immunostimulating complexes. Clinical potential in vaccine development, *Clin. Immunother.* 3 (1995) 461-475.
 - [13] G.F. Rimmelzwaan, A.D.M.E. Osterhaus, A novel generation of viral vaccines based on ISCOM matrix, in: M.F. Powell, M.J. Newman (Eds.), *Vaccine Design, the subunit and adjuvant approach*, Plenum Press, 1995, pp. 543-558.
 - [14] K. Dalsgaard, A study of the isolation and characterization of the saponin Quil A, *Acta Vet. Scand. Suppl.* 69 (1978) 1-40.
 - [15] K. Dalsgaard, L. Hilgers, G. Trouve, Classical and new approaches to adjuvant use in domestic food animals, *Advan. Vet. Sci. Compar. Med.* 35 (1990) 121-160.
 - [16] T. Kartnig von, C.Y. Ri, *Dünnschichtchromatographische Untersuchungen an den Saponinen aus Cortex quillajae*, *Planta Med.* 23 (1973) 269-271.
 - [17] R. Higuchi, Y. Tokimitsu, T. Fujioka, T. Komori, T. Kawasaki, D.G. Oakenfull, Structure of the desacylsaponins obtained from the bark of *Quillaja saponaria*, *Phytochem.* 26 (1987) 229-235.
 - [18] L. Maharaj, K.J. Froth, J.B. Campbell, Immune responses of mice to inactivated rabies vaccine administered orally: potentiation by *Quillaja saponia*, *Can. J. Microbiol.* 32 (1986) 414-420.
 - [19] C. Kensil, C. Patel, M. Lennick, D. Marciani, Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria molina* cortex, *J. Immunol.* 146 (1991) 431-437.
 - [20] C.A. Kensil, D.J. Marciani, G.A. Beltz, C.-H. Hung, Saponin adjuvant, Patent application WO 88/09336, 1988.
 - [21] G.F.A. Kersten, T. Teerlink, H.J.G.M. Derks, A.J. Verkleij, T.L. van Wezel, D.J.A. Crommelin, E.C. Benveny, Incorporation of the major outer membrane protein of *Neisseria Gonorrhoea* in saponin-lipid complexes (ISCOMs): Chemical analysis, some structural features and comparison of their immunogenicity with other antigen delivery systems, *Infect. Immun.* 56 (1988) 432-438.
 - [22] G. Kersten, Aspects of ISCOMS, Analytical, pharmaceutical and adjuvant properties, Dep. Pharmaceutics, University of Utrecht, The Netherlands, Ph.D. thesis, 1990.
 - [23] H.-S. So, H.-S. Yoon, D.-Y. Choi, Y.-S. Kwon, J.-H. Sung, T.G. Lee, E.-S. Park, H.-S. Cho, B.M. Lee, J.M. Cho, W.-S. Ryu, Effect of a novel saponin adjuvant derived from *Quillaja saponaria* on the immune response to recombinant Hepatitis B surface antigen, *Mol. Cells* 7 (1997) 178-186.
 - [24] C.A. Kensil, S. Soltysik, U. Patel, D.J. Marciani, Structure/function relationship in adjuvants from *Quillaja saponaria* Molina, in: F. Brown, R.M. Channock, H.S. Ginsberg, R.A. Lerner, (Eds.), *Cold Spring Harbor Press. Cold Spring Harbor, New York*, 1992, pp. 35-40.
 - [25] R. Higuchi, Y. Tokimitsu, T. Komori, An acylated triterpenoid saponin from *Quillaja saponaria*, *Phytochem.* 27 (1988) 1165-1168.
 - [26] B. Morein, K. Lövgren, K. Daalsgaard, J. Thurin, B. Sundquist, Matrix with immunomodulating activity, Patent application WO 89/00528, 1989.
 - [27] B. Rönberg, M. Fekadu, B. Morein, Adjuvant activity of non-toxic *Quillaja saponaria* Molina components for use in ISCOM-Matrix, *Vaccine* 13 (1995) 1375-1382.
 - [28] S. Behboudi, B. Morein, M. Villacres-Eriksson, In vitro activation of antigen-presenting cells (APC) by defined composition of *Quillaja saponaria* Molina triterpenoids, *Clin. Exp. Immunol.* 105 (1996) 26-30.
 - [29] K. Dalsgaard, M. Henry, R.M. San Martin Gamboa, H.J. Grande, S. Kamstrup, Compounds with adjuvant activity, Patent application WO 95/09179, 1995.
 - [30] N.E. Jacobsen, W.J. Fairbrother, C.R. Kensil, A. Lim, D.A. Wheeler, M.F. Powell, Structure of the saponin adjuvant QS-21 and its base-catalysed isomerization product by ^1H and natural abundance ^{13}C NMR spectroscopy, *Carbohydr. Res.* 280 (1996) 1-14.
 - [31] R. Bomford, M. Stapleton, S. Winsor, J.E. Beesley, E.A. Jessup, K.R. Price, G.R. Fenwick, Adjuvant activity and iscom formation by structurally diverse saponins, *Vaccine* 10 (1992) 572-577.
 - [32] S. Guo, L. Kenne, L.N. Lundgren, B. Rönberg, B. Sundquist, Structural studies of triterpenoid saponins from *Quillaja saponaria* Molina, submitted for publication.
 - [33] S. Soltysik, J.-Y. Wu, J. Recchia, D.A. Wheeler, M.J. Newman, R.T. Coughlin, C.R. Kensil, Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function, *Vaccine* 13 (1995) 1403-1410.
 - [34] C.A. Kensil, S. Soltysik, D.J. Marciani, Saponin-antigen conjugates and the use thereof, Patent application, WO 93/05789, 1993.
 - [35] S. Soltysik, D.A. Bedore, C.R. Kensil, Adjuvant activity of QS-21 isomers, *Ann. New York Acad. Sci.* 690 (1993) 392-395.
 - [36] J.L. Cleland, C.R. Kensil, A. Lim, N.E. Jacobsen, L. Basa, M. Spellum, D.A. Wheeler, J.-Y. Wu, M.F. Powell, Isomerization and formulation stability of the vaccine adjuvant QS-21, *J. Pharm. Sci.* 85 (1996) 22-28.
 - [37] D.J. Pillion, J.A. Amsden, C.R. Kensil, J. Recchia, Structure-function relationship among *Quillaja* saponins serving as excipients for nasal and ocular delivery of insulin, *J. Pharm. Sci.* 85 (1996) 518-524.

- [38] J.C. Cox, A. Coulter, R. Macfarlan, L. Beezum, J. Bates, T.-Y. Wong, Drane, D. Development of an influenza-Iscom vaccine, in: G. Gregoriadis (Ed.), *Vaccine Design: The role of cytokine networks*, Plenum Press, New York, 1997, in press.
- [39] J. Rhodes, Evidence for the intracellular covalent reaction essential in antigen specific T cell activation, *J. Immunol.* 143 (1989) 1482–1489.
- [40] J. Rhodes, H. Chen, S.R. Hall, J.E. Beesley, D.C. Jenkins, P. Collins, B. Zheng, Therapeutic potentiation of the immune system by costimulatory schiff-base-forming drugs, *Nature* 377 (1995) 71–74.
- [41] F.R. Vogel, M.F. Powell, A compendium of vaccine adjuvants and excipients, in: M.F. Powell, M.J. Newman (Eds.), *Vaccine Design*, Plenum Publishing Corporation, New York, 1995, pp. 141–228.
- [42] C.R. Kensil, J.-Y. Wu, S. Soltysik, Structural and immunological characterization of vaccine adjuvant QS-21, in: M.F. Powell, M.J. Newman (Eds.), *Vaccine Design*, Plenum Publishing Corporation, New York, 1995, pp. 525–541.
- [43] B. Sundquist, K. Lövgren, S. Höglund, B. Morein, Influenza virus iscoms: biochemical characterization, *Vaccine* 6 (1988) 44–58.
- [44] R.R. Dourmashkin, R.M. Dougherty, R.J.C. Harris, Electron microscopic observations of Raus sarcoma virus and cell membranes, *Nature* 194 (1962) 1116–1119.
- [45] A.D. Bangham, R.W. Horne, Action of saponin on biological membranes, *Nature* 196 (1962) 953–955.
- [46] A.M. Glaucert, J.T. Dingle, S.A. Lucy, Action of saponin on biological cell membranes, *Nature* 196 (1962) 953–955.
- [47] J.A. Lucy, A.M. Glaucert, Structure and assembly of macromolecular lipid complexes composed of globular micelles, *J. Mol. Biol.* 8 (1964) 727–748.
- [48] M. Horzinek, M. Mussgay, Studies on the substructure of togaviruses. Effect of urea, deoxycholate and saponin on the Sinus virion, *Arch. Gesamte. Virusforsch.* 33 (1971) 296–305.
- [49] B. Morein, K. Lövgren, S. Höglund, B. Sundquist, The ISCOM: and immunostimulating complex, *Immunol. Today* 8 (1987) 335–338.
- [50] M. Özel, S. Höglund, H. Gelderblom, B. Morein, Quaternary structure of the immunostimulating complex (ISCOM), *J. Ultrastruct. Mol. Struct. Res.* 102 (1989) 240–248.
- [51] K. Lövgren, B. Morein, The requirement of lipids for the formation of immunostimulating complexes (iscoms), *Biotechnol. Appl. Biochem.* 10 (1988) 161–172.
- [52] G.F.A. Kersten, A. Spiekstra, E.C. Beuvery, D.J.A. Crommelin, On the structure of immune-stimulating saponin-lipid complexes (iscoms), *Biochim. Biophys. Acta* 1062 (1991) 165–171.
- [53] M. Villacrés-Eriksson, Induction of immune response by Iscoms, Dep. Veterinary Microbiology, Swedish Univ. of Agricultural Science, PhD Thesis, 1993.
- [54] K. Weijer, A. Pfauth, R. van Herwijnen, O. Jarret, R.H. Meloen, C. Tomec, A. Osterhaus, Induction of feline leukaemia virus-neutralizing antibodies by peptides derived from the FeLV env gene, *Vaccine* 11 (1993) 946–956.
- [55] B. Mougin, O. Bakouche, D. Gerlier, Humoral immune response elicited in rats by measles viral membrane antigens presented in liposomes and iscoms, *Vaccine* 6 (1988) 445–448.
- [56] I. Classen, A. Osterhaus, The iscom structure as an immune enhancing moiety: experiences in viral systems, *Res. Immunol.* 143 (1992) 531–541.
- [57] B. Morein, K. Simons, Subunit vaccines against enveloped viruses: virosomes, micelles and other protein complexes, *Vaccine* 3 (1985) 83–93.
- [58] M. Trudel, F. Nandon, C. Séguin, G. Boulay, G. Lussier, Vaccination of rabbits with a bovine herpes virus type 1 subunit vaccine: adjuvant effect of iscoms, *Vaccine* 5 (1987) 239–243.
- [59] M. Merza, S. Belak, B. Morein, Characterization of an iscom prepared with envelope glycoproteins of bovine herpes-virus type 1, *J. Vet. Med. B.* 35 (1988) 695–703.
- [60] M. Trudel, F. Nandon, C. Séguin, C. Simard, G. Lussier, Experimental polyvalent iscom subunit vaccine antibodies that neutralize human and bovine respiratory syncytial virus, *Vaccine* 7 (1989) 12–16.
- [61] S. Höglund, K. Dalsgaard, K. Lövgren, B. Sundquist, A. Osterhaus, B. Morein, Iscoms and immunostimulation with viral antigens, in: W. Harris (ed.), *Subcellular Biochemistry*, Plenum, New York, 1989, pp. 39–68.
- [62] P. De Vries, R.S. van Binnendijk, P. van der Marck, A.L. van Wezel, H.A. Voorma, B. Sundquist, F.G.C.M. Uydebaag, A.D.M.E. Osterhaus, Measles virus fusion protein presented in an Immune-stimulating Complex (Iscom) induces haemolysis-inhibiting and fusion inhibiting antibodies, virus specific T cells and protection in mice, *J. Gen. Virol.* 69 (1988) 549–559.
- [63] L. Åkerblom, P. Nara, N. Dunlop, S. Putney, B. Morein, HIV experimental vaccines based on the iscom technology using envelope and gag gene products, *Biotechnol. Ther.* 4 (1993) 145–161.
- [64] S. Pyle, B. Morein, J. Bess, L. Åkerblom, P. Nara, S. Nigida, N. Lerche, P. Fishinger, L. Arthur, Immune response to immunostimulatory complexes (ISCOMs) prepared from human immunodeficiency virus type 1 (HIV-1) or the HIV-1 external envelope glycoprotein, *Vaccine* 7 (1989) 465–473.
- [65] B. Morein, J. Ekström, K. Lövgren, Increased immunogenicity of a non-amphipathic protein (BSA) after inclusion into iscoms, *J. Immunol. Methods* 128 (1990) 177–181.
- [66] K. Heeg, W. Knon, H. Wagner, Vaccination of class I major histocompatibility complex (MHC)-restricted murine C108^T cytotoxic T lymphocytes towards soluble antigens: immunostimulating ovalbumin complexes enter and allow sensitisation against the immunodominant peptide, *Eur. J. Immunol.* 21 (1991) 1523–1527.
- [67] A. Mowat, M.C.I. Donachie, G. Reid, O. Jarret, Immune-stimulating complexes containing Quil A and protein antigen prime class I MHC-restricted T lymphocytes in vivo and are immunogenic by the oral route, *Immunology* 72 (1991) 317–322.
- [68] G. Reid, Soluble proteins incorporate into iscoms after covalent attachment of fatty acid, *Vaccine* 10 (1992) 597–602.

- [69] M. Browning, G.R. Reid, R. Osborne, O. Jarret, Incorporation of soluble antigens into iscoms: HIV gp120 iscoms induce virus neutralizing antibodies, *Vaccine* 10 (1992) 585–590.
- [70] M. Kazanji, F. Laurent, P. Péry, Immune responses and protective effect in mice vaccinated orally with surface sporozoite protein of *Elmeira falciformis* in iscoms, *Vaccine* 12 (1994) 798–804.
- [71] I.R. Pedersen, T.C. Bøg-Hansen, K. Dalsgaard, P.M.H. Heegaard, Iscom immunization with synthetic peptides representing measles virus hemagglutinin, *Virus Res.* 24 (1992) 145–169.
- [72] K. Lövgren, J. Lindmark, R. Pipkorn, B. Morein, Antigenic presentation of small molecules and synthetic peptides conjugated to a preformed iscom as carrier, *J. Immunol. Methods* 98 (1987) 137–143.
- [73] A. Sjölander, K. Lövgren, S. Ståhl, L. Åslund, M. Hansson, P.-Å. Nygren, M. Larsson, M. Hagstedt, B. Wåhlin, K. Berzins, M. Uhlén, B. Morein, P. Perlmann, High antibody responses in rabbits immunized with influenza virus ISCOMs containing a repeated sequence of the *Plasmodium falciparum* antigen Pf155/RESA, *Vaccine* 9 (1991) 443–450.
- [74] M. Larsson, K. Lövgren, B. Morein, Immunopotential of synthetic oligopeptides by chemical conjugation to ISCOMs, *J. Immunol. Methods* 162 (1993) 257–260.
- [75] K. Lövgren, M. Larsson, Conjugation of synthetic peptides to carrier ISCOMs: factors affecting the immunogenicity of the conjugate, *J. Immunol. Methods* 173 (1994) 237–243.
- [76] H.P. Weiss, L. Stütz, H. Becht, Immunogenic properties of ISCOMs prepared with influenza virus glycoprotein, *Arch. Virol.* 114 (1990) 109–120.
- [77] G.F. Rimmelzwaan, H.J. Siebelink, C. Huisman, B. Moss, M.J. Francis, A.D.M.E. Osterhaus, Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation of surface glycoprotein in immune stimulating complexes, *J. Gen. Virol.* 75 (1994) 2097–2102.
- [78] G.B. Lipford, H. Wagner, K. Heeg, Vaccination with immunodominant peptides encapsulated in Quil A-containing liposomes induces peptide-specific primary CD8⁺ cytotoxic T cells, *Vaccine* 12 (1994) 73–80.
- [79] G.B. Lipford, N. Lohm, S. Bauer, K. Heeg, H. Wagner, The immunodominant peptide from listeriolysin in Quil A liposomes vaccinates CD8⁺ cytotoxic T cells and confers protection to infection, *Immunol. Lett.* 40 (1994) 101–104.
- [80] M.J. Newman, J.Y. Wu, B.U. Gardner, K.J. Munroe, D. Leombruno, J. Recchia, C.R. Kensil, R.T. Coughlin, Saponin adjuvant induction of ovalbumin-specific CD8⁺ cytotoxic T lymphocyte responses, *J. Immunol.* 148 (1992) 2357–2362.
- [81] J.L. Cleland, Design and production of single-immunization vaccines using polylactide polyglycolide microsphere systems, in: M.F. Powell, M.J. Newman (Eds.), *Vaccine Design*, Plenum Publishing Corporation, New York, 1995, pp. 439–462.
- [82] J. Hanes, M. Chiba, R. Langer, Polymer microspheres for vaccine delivery, in: M.F. Powell, M.J. Newman (Eds.), *Vaccine Design*, Plenum Publishing Corporation, New York, 1995, pp. 389–412.
- [83] M.F. Powell, J.L. Cleland, D.J. Eastman, A. Lim, K. Murthy, M.J. Newman, J.H. Numborg, K.P. Weissburg, J.C. Vennari, T. Wrin, Immunogenicity and HIV-1 virus neutralization of MN recombinant glycoprotein 120/HIV-1 QS21 vaccine in baboons, *AIDS Res. Hum. Retroviruses* 10(suppl. 2) (1994) 105–108.
- [84] J.C. Cox, R.E. Sparks, I.C. Jacobs, Vaccine preparations, Patent application WO 94/15636 (1994).
- [85] L. Macdonald, M. Kleinig, J.C. Cox, A single dose (two component) experimental influenza vaccine, *Proc. Intl. Symp. Control. Rel. Bioact. Mater.* 24 (1997) 61–62.
- [86] J.R. Egerton, E.A. Laing, C.M. Thorley, Effect of Quil A, a saponin derivative on the response of sheep to alum precipitated *Bacteroides nodosus* vaccines, *Vet. Sci. Commun.* 2 (1978) 247–252.
- [87] C.M. Cameron, F.J. Bester, An improved *Corynebacterium pseudotuberculosis* for sheep, *Onderstepoort J. Vet. Res.* 51 (1984) 263–268.
- [88] P.A. Bachmann, G. Baljer, X. Gmelch, W. Eichhorn, P. Planck, A. Mayr, Vaccination of cows with K-99 and rotavirus antigen, *Zentralbl. Veterinärmed. Reihe, B.* 31 (1984) 660–668.
- [89] J.A. Montaraz, P. Novotny, J. Ivanyi, Identification of a 68kD protective protein antigen from *Bordetella bronchiseptica*, *Infect. Immun.* 47 (1985) 744–751.
- [90] C.R. Kensil, M.J. Newman, R.T. Coughlin, S. Soltysik, D. Bedore, J. Recchia, J.Y. Wu, D.J. Marciani, The use of Stimulon adjuvant to boost immune response, *Vaccine Res.* 2 (1993) 273–281.
- [91] J.E. Kirkley, P.H. Naylor, D.J. Marciani, C.R. Kensil, M. Newman, A.J. Goldstein, QS-21 augments the antibody response to a synthetic peptide vaccine compared to alum, in: A.L. Goldstein, E. Garaci (Eds.), *Combination Therapies*, Plenum Press, New York, 1992, pp. 231–241.
- [92] R. Bomford, Relative adjuvant efficiency of Al(OH)₃ and saponin is related to the immunogenicity of antigen, *Int. Arch. Allergy Appl. Immunol.* 75 (1984) 280–281.
- [93] J.C. Lei, Aluminum hydroxide gel – guidelines for adsorption, *Vaccine* 3 (1985) 154–155.
- [94] J.C. Cox, A.R. Coulter, Adjuvants – a classification and review of their modes of action, *Vaccine* 15 (1997) 248–256.
- [95] L. Chédid, Adjuvants of immunity, *Ann. Immunol. (Inst. Pasteur)* 136D (1985) 283–291.
- [96] A. Sjölander, K. Bengtsson, B. Morein, Kinetics, localization and cytokine profile of T cell responses to immune stimulating complexes (iscoms) containing human influenza virus envelope glycoproteins, *Vaccine* 15 (1997) 1030–1038.
- [97] D.L. Watson, K. Lövgren, N.A. Watson, C. Fussum, B. Morein, S. Höglund, Inflammatory response and antigen localization following immunization with influenza virus ISCOMs, *Inflammation* 13 (1989) 641–649.
- [98] K.L. Bengtsson, A. Sjölander, Adjuvant activity of iscoms: effect of ratio and co-incorporation of antigen and adjuvant, *Vaccine* 14 (1996) 753–760.
- [99] A. Sjölander, K. Lövgren, K.L. Bengtsson, M. Johansson, B. Morein, Kinetics, localization and isotype profile of antibody responses to immune stimulating complexes (iscoms) con-

- aining human influenza virus envelope glycoproteins, *Scand. J. Immunol.* 43 (1996) 164-172.
- [100] D.L. Watson, N.A. Watson, C. Fossum, K. Lövgren, B. Morein, Interactions between immune stimulating complexes (ISCOMs) and peritoneal mononuclear leukocytes, *Microbiol. Immunol.* 36 (1992) 199-203.
- [101] I.J.T.M. Claassen, A.D.M.E. Osterhaus, E. Claassen, Antigen detection in vivo after immunization with different forms of rabies virus antigen: involvement of marginal metallophilic macrophages in the uptake of immune-stimulating complexes, *Eur. J. Immunol.* 25 (1995) 1446-1452.
- [102] G. Krnal, M. Janse, E. Claassen, Marginal metallophilic macrophages in the mouse spleen: effects of neonatal injections of MOMA-1 antibody on the humoral immune response, *Immunol. Lett.* 17 (1988) 139-144.
- [103] M. Villacres-Eriksson, M. Berström-Mollaoglu, H. Kåberg, The induction of cell-associated and secreted IL-1 by iscoms, matrix, or micelles in murine splenic cells, *Clin. Exp. Immunol.* 93 (1993) 120-125.
- [104] M. Bergström-Mollaoglu, K. Lövgren, L. Åkerblom, Antigen-specific increases in the number of splenocytes expressing MHC Class II molecules following restimulation with antigen in various physical forms, *Scand. J. Immunol.* 36 (1992) 565-574.
- [105] M. Villacres-Eriksson, Antigen presentation by naive macrophages, dendritic cells and B cells following exposure to immunostimulating complexes, *Clin. Exp. Immunol.* 102 (1995) 46-52.
- [106] G. Trinchieri, Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity, *Annu. Rev. Immunol.* 13 (1995) 251-276.
- [107] M. Villacres-Eriksson, S. Behrendt, A.J. Morgan, G. Trinchieri, B. Morein, Immunomodulation by quillaja saponaria adjuvant formulations: in vivo stimulation of interleukin 12 and its effects on the antibody response, *Cytokine* 9 (1997) 73-82.
- [108] J.Y. Wu, R.H. Gardner, N.N. Kushner, L.A. Pozzi, C.R. Kensil, P.A. Cloutier, R.T. Coughlin, M.J. Newman, Accessory cell requirements for saponin adjuvant-induced class I MHC antigen-restricted cytotoxic T-lymphocytes, *Cell. Immunol.* 154 (1994) 393-406.
- [109] T.R. Mossman, R.L. Coffman, TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties, *Annu. Rev. Immunol.* 7 (1989) 145-173.
- [110] M. Yamamura, K. Umemura, R.J. Deans, K. Weinberg, T.H. Rea, B.R. Bloom, R.L. Modlin, Defining protective responses to pathogens: cytokine profiles in leprosy patients, *Science* 254 (1991) 277-279.
- [111] E.P. Heinzel, M.D. Sadick, S.S. Mutha, R.M. Locksley, Production of interferon gamma, interleukin 2, interleukin 4 and interleukin 10 by CD4⁺ lymphocytes in vivo during healing and progressive murine leishmaniasis, *Proc. Natl. Acad. Sci. USA* 88 (1991) 7011-7015.
- [112] M. Clerici, D.R. Lucey, J.A. Berzofsky, L.A. Pinto, T.A. Wynn, S.P. Blatt, M.J. Dolan, C.W. Hendrix, S.F. Wolf, G.M. Sheare, Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro, *Science* 262 (1993) 1721-1724.
- [113] D.L. Emery, J.S. Rothel, P.R. Wood, Influence of adjuvants on the production of gamma-interferon and antibody by ovine lymphocytes, *Immunol. Cell. Biol.* 68 (1990) 127-136.
- [114] C. Fossum, M. Bergström, K. Lövgren, D.L. Watson, B. Morein, Effect of Iscoms and their adjuvant moiety (matrix) on the initial proliferation and IL2 responses: comparison of spleen cells from mice inoculated with Iscoms and/or matrix, *Cell. Immunol.* 129 (1990) 414-425.
- [115] M. Villacres-Eriksson, M. Bergström-Mollaoglu, H. Kåberg, B. Morein, Involvement of IL2 and interferon-gamma in the immune response induced by influenza virus iscoms, *Scand. J. Immunol.* 36 (1992) 421-426.
- [116] R.L. Coffman, D.A. Leiman, P. Rothman, Mechanism and regulation of isotype switching, *Adv. Immunol.* 52 (1993) 229-270.
- [117] E.P. Heinzel, R.M. Rerko, F. Ahmed, E. Pearlman, Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice, *J. Immunol.* 155 (1995) 730-739.
- [118] P.A. Scott, W.C. Eaton, X.-D. Gause, B. Hondowicz, Early IL-4 production does not predict susceptibility to *Leishmania major*, *Exp. Parasitol.* 84 (1996) 178-187.
- [119] A. Sjölander, T.M. Baldwin, J.M. Curtis, E. Handman, Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response is required for generation of immunity to leishmaniasis, *J. Immunol.* (1998) in press.
- [120] E.P. Heinzel, M.D. Sadick, B.J. Holaday, R.L. Coffman, R.M. Locksley, Reciprocal expression of interferon-gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets, *J. Exp. Med.* 169 (1989) 59-72.
- [121] K.J. Erb, C. Blank, H. Moll, Susceptibility to *Leishmania major* in IL-4 transgenic mice is not correlated with the lack of a Th1 immune response, *Immunol. Cell. Biol.* 74 (1996) 239-244.
- [122] M. Kopf, F. Brombacher, G. Köhler, G. Kienle, K.-H. Widmann, C. Leifang, B. Humborg, B. Ledermann, W. Seibach, B.-J. deficient Balb/c mice resist infection with *Leishmania major*, *J. Exp. Med.* 184 (1996) 1127-1136.
- [123] L. Morris, A.B. Trout, K.S. McLeod, A. Kelso, E. Handman, T. Aebischer, Interleukin-1 but not gamma interferon production correlates with the severity of murine cutaneous leishmaniasis, *Infect. Immun.* 61 (1993) 3459-3465.
- [124] A. Sjölander, B. van't Land, K.L. Bengtsson, Iscoms containing purified *Quillaja* saponins upregulate both Th1-like and Th2-like immune responses, *Cell. Immunol.* 177 (1997) 69-76.
- [125] K. Maloy, A.M. Donachie, A.M. Mowat, Induction of Th1 and Th2 CD4⁺ T cell responses by oral or parenteral immunization with ISCOMS, *Eur. J. Immunol.* 25 (1995) 2835-2841.
- [126] E. Dotsika, E. Karagouni, B. Sundquist, B. Morein, A. Morgan, M. Villacres-Eriksson, Influence of *Quillaja*

- saponaria* triterpenoid content on the immunomodulatory capacity of Epstein-Barr virus ISCOMs, *Scand. J. Immunol.* 45 (1996) 261-268.
- [127] J.P. Valensi, J.R. Carlson, G.A. van Nest, Systemic cytokine profiles in BALB/c mice immunised with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants, *J. Immunol.* 153 (1994) 4029-4039.
- [128] G.E. Hancock, D.J. Speelman, K. Heers, E. Bortell, J. Smith, C. Cosco, Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus, *J. Virol.* 11 (1996) 7783-7791.
- [129] C.A. Janeway, P. Travers, *Immunobiology* (Ch. 4), Blackwell, Oxford, 1994.
- [130] E. Maraskovsky, W.-F. Chen, K. Shortman, IL-2 and IFN- γ are two necessary lymphokines in the development of cytolytic T cells, *J. Immunol.* 143 (1989) 1210-1214.
- [131] H. Takahashi, T. Takeshita, B. Morein, S. Putney, R.N. Germain, J. Berzofsky, Induction of CD8 $^{+}$ cytotoxic T cells by immunization with purified HIV-1 envelope proteins in iscoms, *Nature* 344 (1990) 873-875.
- [132] R.S. Van Binnendijk, C.A. van Baalen, M.C.M. Poelen, P. de Vries, J. Boes, V. Cerundolo, A.D.M.E. Osterhaus, F.G.C.M. Uytendael, Measles virus transmembrane fusion protein synthesized de novo or presented in iscom is endogenously processed for HLA class I-and class II-restricted T cell recognition, *J. Exp. Med.* 167 (1992) 119-128.
- [133] U. Zugel, B. Schoel, S. Yamamoto, H. Hengel, B. Morein, S.J.I. Kaufman, Crossrecognition by CD8 T cell receptor alpha beta cytotoxic T lymphocytes of peptides in the self and the mycobacterial hsp60 which share intermediate sequence homology, *Eur. J. Immunol.* 25 (1995) 451-458.
- [134] I. Tarpey, S.N. Stacey, A. McIndoe, D.H. Davies, Priming in vivo and quantification in vitro of class I MHC-restricted cytotoxic T cells to human papilloma virus type II early proteins (E6 and E7) using immunostimulating complexes (ISCOMs), *Vaccine* 14 (1996) 230-236.
- [135] E.G. Hulskoete, G.F. Rimmelzwaan, J. Boes, M.L. Bosch, J.L. Heeney, S.G. Norley, P. de Vries, A.D. Osterhaus, Antigenicity and immunogenicity of recombinant enveloped glycoproteins of HIVmac32H with different in vivo passage histories, *Vaccine* 13 (1995) 1187-1197.
- [136] M. Trudel, F. Naudon, C. Séguin, S. Braut, Y. Lussignan, S. Lemieux, Initiation of cytotoxic T-cell response and protection of Balb/c mice by vaccination with an experimental ISCOMs respiratory syncytial virus subunit vaccine, *Vaccine* 10 (1992) 107-112.
- [137] P.D. Jones, R. Tha Hla, B. Morein, K. Lövgren, G.L. Ada, Cellular immune responses in the murine lung to local immunization with influenza A virus glycoproteins in micelles and immunostimulatory complexes (ISCOMs), *Scand. J. Immunol.* 27 (1988) 645-652.
- [138] A. Mowat, K.J. Maloy, A.M. Donachie, Immune stimulating complexes as adjuvants for inducing local and systemic immunity after oral immunization with protein antigens, *Immunology* 80 (1993) 527-534.
- [139] S.C. Hsu, E.B. Schadeck, A. Delmas, M. Shaw, M.W. Steward, Linkage of a fusion peptide to a CTL epitope from the nucleoprotein measles virus enables incorporation into ISCOMs and induction of CTL responses following intranasal immunization, *Vaccine* 14 (1996) 1159-1166.
- [140] J.Y. Wu, B.H. Gardner, C.J. Murphy, J.R. Seals, C.R. Kensil, J. Recchia, G.A. Beltz, G.W. Newman, M.J. Newman, Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine, *J. Immunol.* 148 (1992) 1519-1525.
- [141] M. Shirai, C.D. Pendleton, J. Ahlers, T. Takeshita, M.J. Newman, J.A. Berzofsky, Helper-cytotoxic T lymphocyte (CTL) determinant linkage required for priming of anti-HIV CD8 $^{+}$ CTL in vivo with peptide vaccine constructs, *J. Immunol.* 152 (1994) 549-556.
- [142] W. Britt, J. Fay, J. Seals, C. Kensil, Formulation of an immunogenic human cytomegalovirus vaccine: responses in mice, *J. Infect. Dis.* 171 (1995) 18-25.
- [143] G.E. Hancock, D.J. Speelman, P.J. Frenchick, M.M. Mineo-Kuhn, R.B. Baggs, D.J. Hahn, Formulation of the purified fusion protein of respiratory syncytial virus with the saponin QS-21 induces protective immune responses in Balb/c mice that are similar to those generated by experimental infection, *Vaccine* 13 (1995) 391-400.
- [144] R.G. Fenton, C.J. Keller, N. Hanna, D.D. Taub, Induction of T-cell immunity against Ras oncoproteins by soluble protein or Ras expressing *Escherichia coli*, *J. Natl. Cancer Inst.* 87 (1995) 1853-1861.
- [145] M.J. Newman, K.J. Munroe, C.A. Anderson, C.J. Murphy, D.L. Panicali, J.R. Seals, J.Y. Wu, M.S. Wyand, C.R. Kensil, Induction of antigen-specific killer T lymphocyte responses using subunit HIVmac251 gag and env vaccines containing QS-21 saponin adjuvant, *AIDS Res. Hum. Retroviruses* 10 (1994) 853-861.
- [146] S. Raychaudhuri, M. Tonks, F. Carbone, T. Ryskamp, W.J.W. Morrow, N. Hanna, Induction of antigen specific class I restricted cytotoxic T cells by soluble proteins in vivo, *Proc. Natl. Acad. Sci. USA* 89 (1992) 8308-8312.
- [147] S.B. Dillon, S.G. Demuth, M.A. Schneider, C.B. Weston, C.S. Jones, J.F. Young, M. Scoll, P.K. Bhanaghar, S. LeCaster, N. Hanna, Induction of protective class I MHC-restricted CTL in mice by recombinant influenza vaccine in aluminium hydroxide adjuvant, *Vaccine* 10 (1992) 309-317.
- [148] F. Zhou, L. Huang, Monophosphoryl lipid A enhances specific CTL induction by a soluble protein antigen entrapped in liposomes, *Vaccine* 11 (1993) 1139-1144.
- [149] K. Deres, H. Schild, K.H. Wiesmüller, G. Jung, H.G. Rammensee, In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine, *Nature* 342 (1989) 561-564.
- [150] A.J. George, Legal status and toxicity of saponins, *Food Cosmet. Toxicol.* 3 (1965) 85-91.
- [151] C. Coulson, R. Evans, The effect of saponins, sterols and linoleic acid on weight increase of growing rats, *Br. J. Nutr.* 14 (1960) 121-134.
- [152] R. Tschesche, G. Wulff, Chemie und Biologie der Saponine, in: W. Herz, H. Grisebach, G. Kirby, (Eds.),

- Fortschritte der chemie organischer Naturstoffe*, Vol. 30, Springer-Verlag, Wien/New York, 1996, pp. 461–496.
- [153] L.M. Flebbe, H. Bradley-Mullen, Immunopotentiating effects of the adjuvants SGP and Quil A, *Cell. Immunol.* 99 (1986) 119–127.
- [154] F. Stieckner, G. Kersten, J. van Bloois, D.J.A. Crommelin, S.L. Hem, J. Lower, J. Kreuter, Comparison of 24 different adjuvants for inactivated HIV-2 split whole virus as antigen in mice. Induction of titres of binding antibodies and toxicity of the formulations, *Vaccine* 13 (1995) 45–53.
- [155] A. Osterhaus, K. Weijer, F. Uytendaele, P. Knell, O. Jarret, L. Åkerblom, B. Morein, Serological responses in cats vaccinated with FeLV iscom and an inactivated FeLV vaccine, *Vaccine* 7 (1989) 137–141.
- [156] B.G. Sundquist, G. Czifra, L. Stipkovits, Protective immunity induced in chicken by a single immunization with *Mycoplasma gallisepticum* immunostimulating complexes (ISCOMS), *Vaccine* 14 (1996) 892–897.
- [157] N. Clark, N.N. Kushner, C.B. Barrett, C.R. Kensil, D. Salisbury, S. Cotter, Efficacy and safety field trials of a recombinant DNA vaccine against feline leukemia virus infection, *J. Am. Vet. Med. Assoc.* 199 (1991) 1433–1443.
- [158] J. Ma, P.A. Bulger, S. Dante, D. van Riet Davis, B. Perilli-Palmer, R.T. Coughlin, Characterization of canine humoral immune responses to outer surface protein subunit vaccines and to natural infection by Lyme disease spirochetes, *J. Infect. Dis.* 171 (1995) 909–915.
- [159] P. De Vries, J.L. Heeney, J. Boes, M.E.M. Dings, F.G.J. Hulskotte, R. Dubbes, W. Koornstra, P. Haafi, L. Åkerblom, S. Eriksson, B. Morein, S. Norley, A.D.M.E. Osterhaus, Protection of rhesus macaques from SIV infection by immunization with different experimental SIV vaccines, *Vaccine* 12 (1994) 1443–1454.
- [160] B.A. Vanselow, I. Abetz, K. Trenfield, A bovine ephemeral fever vaccine incorporating adjuvant Quil A: A comparative study using adjuvants Quil A, aluminium hydroxide gel and dextran sulphate, *Vet. Rec.* 117 (1985) 37–43.
- [161] G.J.A. Speijers, L.H.J.C. Danse, E.C. Beuvery, J. Strik, J.G. Vos, Local reactions of the saponin Quil A containing iscom measles vaccine after intramuscular injection of rats: A comparison with the effect of DPT-Polio vaccine, *Fundam. Appl. Toxicol.* 10 (1988) 425–430.
- [162] A.C. Allison, N.E. Byars, An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell immunity, *J. Immunol. Meth.* 95 (1986) 157–168.
- [163] H.A.C. Atkinson, I.T. Johnson, J.M. Gee, F. Grigoriadou, K. Miller, Brown Norway rat model of food allergy: effect of plant components on the development of oral sensitization, *Food Chem. Toxicol.* 34 (1996) 27–32.
- [164] L. Black, The adjuvant activity of saponin and aluminium hydroxide for promoting reagins, *J. Immunol. Meth.* 15 (1977) 193–195.
- [165] L.S. Kind, C. Ramaika, E. Allaway, Antigenic, adjuvant and permeability enhancing properties of melittin in mice, *Allergy* 36 (1981) 155–160.
- [166] J. Bates, J. Ackland, A. Coulter, J. Cox, D. Drane, R. Macfarlan, J. Varigos, T.-Y. Wong, W. Woods, IscomTM adjuvant – a promising adjuvant for influenza virus vaccines, in: L.E. Brown, A.W. Hampson, R.G. Webster (Eds.), *Options for the Control of Influenza III*, Elsevier, Amsterdam, 1996, pp. 661–667.
- [167] J.C. Cox, A.R. Coulter, Advances in adjuvant technology and applications. In: W.K. Wong (Ed.), *Animal Parasite Control Utilizing Technology*, CRC Press, Boca Raton, 1992, pp. 49–112.
- [168] E.E. Karagouni, J. Hadjipetrou-Kourounakis, Regulation of isotype immunoglobulin production by adjuvants in vivo, *Scand. J. Immunol.* 31 (1990) 745–754.
- [169] L.M. Flebbe, H. Bradley-Mullen, Immunopotentiality by SGP and Quil A, *Cell. Immunol.* 99 (1986) 128–139.
- [170] A.C. White, P. Cloutier, R.T. Coughlin, A purified saponin acts as an adjuvant for a T-independent antigen, in: M.Z. Atassi, (Ed.), *Plenum Press*, New York, 1991, pp. 207–210.
- [171] R.T. Coughlin, C. Chu, A. Fattom, A.C. White, S. Winston, Adjuvant activity of QS-21 for experimental *E. coli* 018 polysaccharide vaccines, *Vaccine* 13 (1995) 17–21.
- [172] A. Fattom, X. Li, Y.H. Cho, A. Burns, A. Hawwari, S.E. Shepherd, R. Coughlin, S. Winston, R. Naso, Effect of conjugation methodology, carrier protein and adjuvants on the immune response to *Staphylococcus aureus* capsular polysaccharide, *Vaccine* 13 (1995) 1288–1293.
- [173] P.O. Livingston, R. Koganty, B.M. Longnecker, K.O. Lloyd, M. Calves, Studies on the immunogenicity of synthetic and natural Thomsen–Friedenreich (TF) antigens in mice: Augmentation of the response by Quil A and Saf-M adjuvants and analysis of the specificity of responses, *Vaccine Res.* 1 (1992) 99–109.
- [174] A. Osterhaus, P. de Vries, B. Morein, L. Åkerblom, J. Heeney, SIV-specific protective immunity in macaques vaccinated with SIV iscom or MDP adjuvanted whole inactivated SIV. Presentation de Colloque de Cent Gardes, 28–30 October 1991, Marnes-la-Coquette, France, *Proceedings* pp. 277–280. *AIDS Res. Hum. Retroviruses* 8 (1992) 1507–1510.
- [175] M. Lavi, U. Rudén, D. Birc, L. Loomis, R. Redfield, K. Lövgren, L. Åkerblom, E. Sandström, B. Wahren, Effects of adjuvants and multiple antigen peptides on humoral and cellular immune responses to gp160 of HIV-1, *J. Acquir. Immune Defic. Syndr.* 6 (1993) 855–864.
- [176] E. Puentes, E. Cangeio, A. Erias, Efficiency of various non-oily adjuvants in immunization against Aujeszky's Disease (Pseudorabies) virus, *J. Vet. Med. B.* 40 (1993) 353–365.
- [177] E.T.S. Ben Ahmed, R. Jennings, M. Ertek, C.W. Potter, The IgA and subclass IgG responses and protection in mice immunised with influenza antigens administered as iscoms, with FCA, ALH or as infectious virus, *Arch. Virol.* 125 (1992) 71–86.
- [178] E.T.S. Ben Ahmed, G. Gregoriadis, C.W. Potter, R. Jennings, Immunopotentiality of local and systemic immune responses by ISCOMs, liposomes and FCA: role in protection against influenza A in mice, *Vaccine* 11 (1993) 1302–1309.

- [179] K. Lövgren, H. Kåberg, B. Morein, An experimental influenza subunit vaccine (ISCOM) – induction of protective immunity to challenge infection in mice after intranasal or subcutaneous administration, *Clin. Exp. Immunol.* 82 (1990) 435–439.
- [180] M. Kazanji, F. Laurent, P. Péry, Immune responses and protective effect in mice vaccinated orally with surface sporozoite protein of *Elmeira falciformis* in iscoms, *Vaccine* 12 (1994) 798–804.
- [181] H.O. Ghazi, C.W. Potter, T.L. Smith, R. Jennings, Comparative antibody responses and protection in mice immunised by oral or parenteral routes with influenza virus subunit antigens in aqueous form or incorporated into ISCOMS, *J. Med. Microbiol.* 42 (1995) 53–61.
- [182] L.A. Van Pinxtere, I. Campbell, C.J. Clarke, D.R. Snodgrass, M.G. Bruce, A single oral dose of inactivated rotavirus and ISCOM matrices induces partial protection in lambs, *Biochem. Soc. Trans.* 25 (1997) 340S.
- [183] K. Robinson, T. Bellaby, D. Wakelin, Vaccination against the nematode *Trichinella spiralis* in high and low responder mice. Effects of different adjuvants, protective immunity and immune response, *Immunology* 82 (1994) 261–267.
- [184] D.J. Pillion, J.A. Atchinson, C. Garguilo, R.-X. Wang, P. Wang, E. Meezan, Efficacy of insulin in nosedrops: New formulations containing alkylglycosides, *Endocrinol.* 135 (1994) 2386–2391.
- [185] P. Albrecht, F.A. Ennis, E.J. Salzman, S. Krugman, Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure, *J. Pediatr.* 91 (1977) 715–718.
- [186] R.S. van Binnendijk, C.M. Poolen, G. van Amerongen, P. de Vries, A.D.M.E. Osterhaus, Protective immunity in Macaques vaccinated with live attenuated recombinant and subunit measles vaccines in the presence of passively acquired antibodies, *J. Infect. Dis.* 175 (1997) 524–532.
- [187] A. Nordengrahn, M. Rusvai, M. Merza, J. Ekström, B. Morein, S. Belak, Equine herpesvirus type 2 (EHV-2) as a predisposing factor for *Rhodococcus equi* pneumonia in foals: prevention of the bifactorial disease with EHV-2 immunostimulating complexes, *Vet. Microbiol.* 51 (1996) 55–68.
- [188] C.R. Kensil, C. Barrett, N. Kushner, G. Beltz, J. Storey, U. Patel, J. Recchia, A. Aubert, D. Marciani, Development of a genetically engineered vaccine against feline leukemia virus infection, *J. Am. Vet. Med. Assoc.* 199 (1991) 1423–1427.
- [189] D.J. Marciani, C.R. Kensil, G.A. Beltz, G.A. Hung, J. Cronier, A. Aubert, Genetically-engineered subunit vaccine against feline leukemia virus: protective immune response in cats, *Vaccine* 9 (1991) 89–96.
- [190] R.T. Coughlin, D. Fish, T.N. Mather, J. Ma, C. Pavia, P. Bulger, Protection of dogs from Lyme disease with vaccine containing outer surface protein (OspA), OspB and the saponin adjuvant QS-21, *J. Infect. Dis.* 171 (1995) 1049–1052.
- [191] K. Kitamura, P.O. Livingston, S.R. Fortunato, E. Stockert, F. Helling, G. Ritter, H.F. Oettgen, L.J. Old, Serological response patterns of melanoma patients immunized with a GM2 ganglioside conjugate vaccine, *Proc. Natl. Acad. Sci. USA*, 92 (1995) 2805–2809.
- [192] P.O. Livingston, S. Adluri, F. Helling, T.J. Yao, C.R. Kensil, M.J. Newman, D. Marciani, Phase I trial of immunological adjuvant QS-21 with GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma, *Vaccine* 12 (1994) 1275–1280.
- [193] P. Livingston, S. Zhang, S. Adluri, T.J. Yao, L. Gracber, G. Ragupathi, F. Helling, M. Fleisher, Tumor cell reactivity mediated by IgM antibodies in sera from melanoma patients vaccinated with GM2 ganglioside covalently linked to KLH is increased by IgG antibodies, *Cancer Immunol. Immunother.* 43 (1997) 324–330.



A service of the National Library of Medicine
and the National Institutes of Health

My NCBI
[Sign In] [Register]

All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Books

Search PubMed for [] Go Clear

Limits Preview/Index History Clipboard Details

Display AbstractPlus Show 20 Sort By Send to

All: 1 Review: 0

1: J Immunol Methods. 1987 Apr 2;98(1):137-43.

Links

Antigenic presentation of small molecules and peptides conjugated to a preformed iscom as carrier.

Lövgren K, Lindmark J, Pipkorn R, Morein B.

The aim of the present study was to elaborate a carrier system for haptens and synthetic peptides, making them immunogenic without addition of Freund's adjuvants. As carriers, preformed iscoms and micelles as well as BSA have been compared. The iscoms and micelles were prepared with envelope proteins of an influenza virus. As a model hapten, the small molecules of biotin were coupled to iscoms to determine the optimum epitope density for induction of an enhanced antibody response to the hapten. The most efficient carrier tested was the preformed iscom at an epitope density of ten biotin molecules per viral protein in the iscom. This carrier system exceeded the efficacy of both the preformed micelles and BSA, the latter with or without addition of Freund's adjuvant. A favourable epitope density could not be achieved when each of two different synthetic peptides was conjugated to iscoms. Epitope densities higher than one to three peptide molecules per protein lead to polymerization of either the peptide or the carrier. The coupling agent was glutaraldehyde.

PMID: 2435808 [PubMed - indexed for MEDLINE]

Display AbstractPlus Show 20 Sort By Send to

Related Links

Induction of feline leukaemia virus-neutralizing antibodies by immunization with synthetic peptides derived from the FeLV envelope. [Vaccine. 1993]

Photochemical linking of primary aromatic amines to carrier proteins to elicit antibody response against the amine haptens. [J Immunol Methods. 1986]

Conjugation of synthetic peptides to carrier iscoms: factors affecting the immunogenicity of the conjugate. [J Immunol Methods. 1994]

Immunopotential of synthetic oligopeptides by chemical conjugation to iscoms. [J Immunol Methods. 1993]

Peptide polymerisation facilitates incorporation into ISCOMs and increases antigen-specific IgG2a production. [Vaccine. 1995]

See all Related Articles...

Write to the Help Desk

NCBI | NLM | NIH

Department of Health & Human Services

Privacy Statement | Freedom of Information Act | Disclaimer